IMMUNOPATHOLOGY OF *CHLAMYDOPHILA ABORTUS* INFECTION IN A PREGNANT MOUSE MODEL

KAREN KERR

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DECLARATION

The work reported in this thesis was carried out under the supervision of Dr David Longbottom and Dr Gary Entrican at the Moredun Research Institute and Professor Declan McKeever at the Centre for Tropical Veterinary Medicine, University of Edinburgh. All results present, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

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ABBREVIATIONS

ABC	Avidin-biotin complex
ADCC	Antibody-dependent cellular cytotoxicity
ANOVA	Analysis of Variance
ATP	Adenosine tri-phosphate
ADP	Adenosine di-phosphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
CFT	Complement fixation test
COMC	Chlamydial outer membrane complex
Con A	Concanavilin A
Cpm	Counts per minute
CSF	Colony-stimulating factor
CTL	Cytotoxic lymphocytes
CTM	Chlamydial transport medium
DAB	Diaminobenzidine tetrahydrochloride
dH_2O	Distilled water
DIG	Digoxygenin
dNTPs	Deoxynucleoside 5´-triphosphates
DNA	Deoxyribonucleic acid
Dpc	Day post-coitum
DTH	Delayed type hypersensitivity

EB	Elementary body
EAE	Enzootic abortion of ewes
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetra-acetic acid
FBS	Foetal bovine serum
fc	final concentration
FcR	Fc receptor
FLC	Flow cytometry
g	Gram
xg	G-force
GMG	Granulated metrial gland
GLM	General linear model
H &E	Haematoxylin & Eosin
h	Hours
HEPES	N-2-Hydroxyethylpiperazine-N´-2-ethane-sulphonic acid
HK	Heat-killed
HRP	Horse-radish peroxidase
HSB	High salt buffer
IDO	Indolamine 2,3-dioxygenase
IFU	Inclusion forming units
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin

i.p.	Intra-peritoneal
IPTG	Isopropyl-β-D-thiogalactoside pyranoside
kD	Kilo Dalton
KO	Knockout
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
MHC	Major histocompatability complex
MOMP	Major outer membrane protein
mRNA	Messenger ribonucleic acid
min	Minute
mAb	Monoclonal antibody
MRI	Moredun Research Institute
MoPn	Mouse pneumonitis
MWM	Mouse wash medium
NBT	Nitroblue tetrazolium
NFDM	Non-fat dried milk
NK	Natural killer
OD	Optical density
OEA	Ovine enzootic abortion
Omp	Outer membrane protein
OPD	o-phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PBST20	PBS containing Tween 20
PBST80	PBS containing Tween 80
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophils
pmp	Polymorphic membrane protein
POMP	Polymorphic outer membrane protein
p.i.	Post-infection
PGE_2	Prostaglandin E_2
POMPs	Putative outer membrane proteins
RB	Reticulate body
RFLP	Restriction fragment length polymorphism
RT	Room temperature
s	Seconds
SDS	Sodium dodecyl sulphate
SIs	Stimulation indices
SSC	Saline-sodium citrate buffer
STD	Sexually transmitted disease
STSM	Short Term Scientific Mission
TCR	T cell receptor
Th	T helper cell
TBS	Tris-buffered saline
TBST	TBS containing 0.1% Tween 20
TNF-α	Tumour necrosis factor alpha
Tris	Tris [hydroxymethyl] aminomethane
T/V	Trypsin/versene
TX	Triton-X-100

U	Unit (s)
V	Volts
v/v	Volume/volume
WT	Wild-type
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-thiogalactopyranoside
ZSF	Zinc salts fixative

ABSTRACT

Chlamydophila abortus targets the ovine placenta, causing tissue damage, inflammation and abortion. *C. abortus* is the main infectious cause of abortion in ewes in the UK and results in major economic losses to the sheep industry. A pregnant mouse model was developed to investigate immune responses and disease pathogenesis for comparison with the ovine disease.

Pregnant mice were inoculated at mid-gestation with *C. abortus* to investigate progression and pathogenesis of infection. This resulted in abortion on days 6-8 post-infection (p.i.). Infected cells were identified at the maternal-foetal interface on days 3 and 5 p.i. and chlamydial inclusions were scattered throughout the trophoblastic labyrinth of the placenta between days 3 and 7 p.i.. Infected areas were accompanied by a maternal mononuclear inflammatory cellular infiltrate, including polymorphonuclear neutrophils, B cells and CD4 and CD8 T cells. *C. abortus* organisms were cultured from both maternal and foetal tissues, higher numbers present in placenta, the target organ.

A Th1 type immune response was characterised in the mouse model, similar to that in ovine infections. A dominant IgG2a antibody response was identified and IFN-γ and TNF-α expression were detected in both sera and supernatants from stimulated splenocytes. IFN-γ mRNA and TNF-α mRNA expression were detected by *in situ* hybridisation in mouse tissues infected with *C. abortus*. A latent/subclinical persistent infection did not appear to develop in non-pregnant mice infected with *C. abortus* and abortion did not occur in the subsequent pregnancy, in contrast to that in ovine infections. Repeat abortion did not occur in the pregnancy subsequent to abortion in mice, similar to the situation observed in sheep. Mice were also immune to secondary infection in the pregnancy subsequent to abortion.

Infection of pregnant mice resulted in abortion as observed in infected ewes, and a similar Th1 immune response is elicited in both sheep and mice. This model will allow the rapid screening of novel protein and DNA based vaccines to protect against chlamydial abortion.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction to Chlamydiae

Chlamydiae are obligate intracellular bacteria that infect epithelial cells and monocyte/macrophages of a wide host range resulting in a broad spectrum of diseases (summarised in **Table 1.1**). *Chlamydia trachomatis* is the world's leading cause of preventable blindness and sexually transmitted disease in man. *Chlamydophila pneumoniae* can cause respiratory infections and may lead to chronic diseases, such as atherosclerosis and arthritis. Animal infections vary from abortion in ruminants and pigs as a result of *Chlamydophila abortus* infection, to conjunctivitis in guinea pigs following infection with *Chlamydophila caviae,* which acts as a key model for *C. trachomatis* infections. Guinea pigs with primary infections develop immunity to reinfection of the eyes or the genital tract (Ahmad *et al.*, 1977; Mount *et al.*, 1973) and this showed that both humoral and cell-mediated immunity are required for resolution of genital tract infection (Rank *et al.*, 1981; Rank & Batteiger, 1989; Rank *et al.*, 1989).

Chlamydophila psittaci is a worldwide pathogen of both wild and domesticated birds (Grimes & Wyrick, 1991; Meyer, 1967) causing acute, subacute, chronic or subclinical infections of the respiratory system. Both animal pathogens *C. abortus* and *C. psittaci* are zoonotic, meaning infection of humans is possible, the latter resulting in psittacosis, which is the most important animal chlamydiosis transmissible to man (Longbottom & Coulter, 2003). *C. abortus* can infect pregnant women and this can be life threatening to both the mother and the foetus (Buxton, 1986; Hyde & Benirschke, 1997; Meijer *et al.*, 2004), although infection of women is relatively rare compared to avian chlamydiosis, which is a significant occupational health risk to those people working in the poultry and pet industries (Vanrompay *et al.*, 1995).

The family *Chlamydiaceae* was classified as four different species within one genus, *Chlamydia.* Following re-classification in 1999, the order *Chlamydiales* now comprises four distinct families, the *Simkaniaceae, Parachlamydiaceae, Waddliaceae* and *Chlamydiaceae* (Everett *et al.*, 1999a). The family *Chlamydiaceae*

is divided into two genera, *Chlamydia* and *Chlamydophila* and nine species (**Table 1.1**). This distinction is based on limited phenotypic, morphological and genetic criteria (Corsaro *et al.*, 2003; Everett *et al.*, 1999a).

Members of the family *Chlamydiaceae* have greater than 90% 16S rRNA identity. The complete 16S and 23S rRNA genes of *Chlamydophila* are ≥95% identical. This genus is readily identified and distinguished from the other genus by ribosomal signature sequences, or by inspection of the ribosomal intergenic spacer (Everett & Andersen, 1999). The nomenclature proposed by Everett and Andersen (Everett & Andersen, 1999) has not been completely accepted in the chlamydial field (Everett & Andersen, 2001; Schachter *et al.*, 2001) as suggested by its lack of use in the literature, particularly by the human research community. However, those working in the animal research field have adopted this new nomenclature, as it has reduced much confusion. The new classification will be adopted throughout this thesis.

Table 1.1 The Family *Chlamydiaceae* (Kerr *et al.*, 2005)

Species	Host	Disease/symptoms
Chlamydia trachomatis	Humans	Chronic conjunctivitis (trachoma) Sexually transmitted disease; pelvic inflammatory disease; infertility
Chlamydia muridarum	Mice, hamsters	Respiratory and genital tract infection (model for *C. trachomatis* infection in humans)
Chlamydia suis	Pigs	Intestinal, respiratory and reproductive disease
Chlamydophila pneumoniae	Humans, koalas, reptiles, amphibians	Pneumonia, bronchitis, pharyngitis; also associated with atherosclerosis, reactive arthritis and asthma
Chlamydophila psittaci	Birds, poultry	Respiratory disease (zoonotic pathogen)
Chlamydophila abortus	Ruminants, pigs	Abortion (zoonotic pathogen)
Chlamydophila pecorum	Ruminants, pigs, koala	Enteric disease; pneumonia; conjunctivitis; polyarthritis; metritis and encephalomyelitis
Chlamydophila felis	Cats	Conjunctivitis (zoonotic pathogen)
Chlamydophila caviae	Guinea pig	Ocular and genital tract infection (model for *C. trachomatis* infection in humans)

Chlamydiae are deeply separated from other bacteria on a phylogenetic scale, which forms the basis for a distinct bacterial division and they are themselves exceptionally diverse (Thomas *et al.*, 1997). The chlamydial envelope has similarities with that of other Gram-negative organisms: an inner membrane, a periplasmic space and an outer membrane containing proteins and lipopolysaccharide (LPS) (Stephens & Lammel, 2001). However, differences to other Gram-negative bacteria include the structure of LPS; in *Chlamydia*, it is truncated, terminating in 3-deoxy-D-manno-octulosonic acid (Stephens & Lammel, 2001). Chlamydiae have little or no detectable peptidoglycan, although a full set of peptidoglycan synthesis genes has been identified following genome analysis (Stephens *et al.*, 1998). One of the main problems of molecular research into this intracellular pathogen is the lack of a suitable gene transfer system. However, the identification of a cryptic plasmid in

Chlamydia species may provide a vector with a potential region for the insertion of foreign DNA, to allow transformation (Thomas *et al.*, 1997).

1.2 Developmental cycle of *Chlamydia*

Chlamydiae undergo a biphasic developmental cycle (**Figure 1.1**) that consists of two distinct morphological forms, the elementary body (EB) and the reticulate body (RB), which are specifically adapted to extracellular and intracellular environments respectively (Longbottom & Coulter, 2003). The EB, which is the infectious form, is small in size (200-300 nm), dense, metabolically inactive and stable within the extracellular environment. RBs are noninfectious, larger in size (500-1000 nm), metabolically active and less electron-dense than EBs.

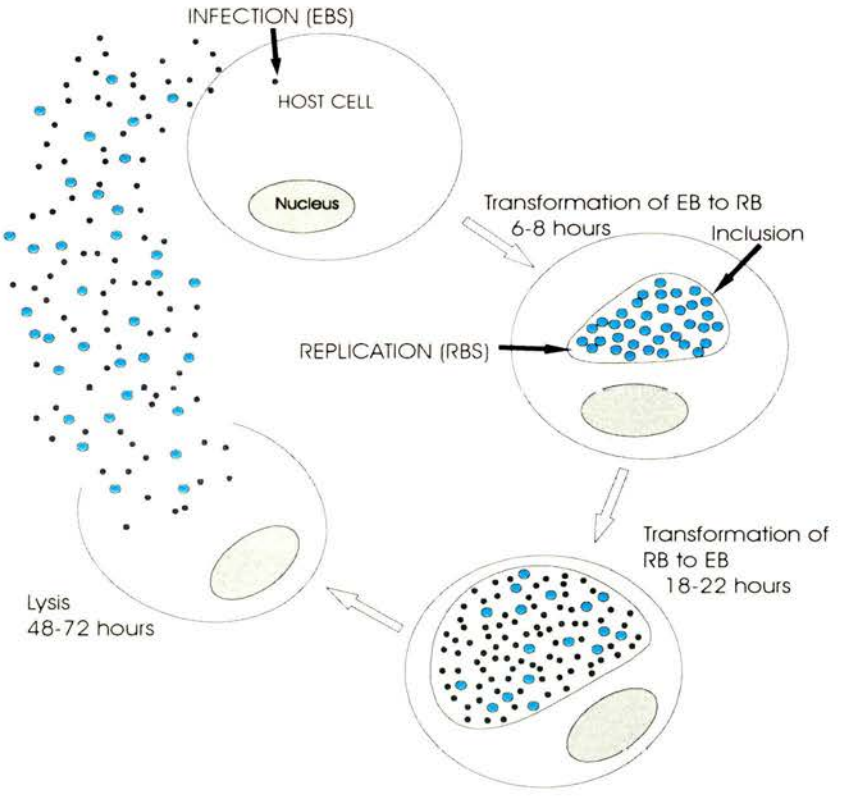


Figure 1.1 Basic developmental cycle of the Chlamydiales (adapted from (Everett, 2000))

EBs give rise to primary infection of susceptible hosts by attaching to the cytoplasmic membrane of mucosal epithelial cells. EBs are endocytosed into a membrane-bound vacuole, called the chlamydial inclusion (Field & Barnes, 1992). Despite their lack of petidoglycan, EBs have a rigid cell wall structure, which is due to the presence of a major protein (MOMP – major outer membrane protein) cross-linked with other cysteine-rich proteins present in the cell envelope (Everett & Hatch, 1991). The EB is highly resistant to lysis due to this complex of outer membrane proteins cross-linked by disulphide bonds, protecting the bacterium from otherwise damaging extracellular environmental factors (Stephens & Lammel, 2001).

Upon entry, the EB, which resides within the intracytoplasmic inclusion, transforms into the larger, non-infectious RB approximately 6-8 hours post-infection (p.i.). The RB is metabolically active and is capable of synthesizing its own macromolecules. Until recently, chlamydiae were thought to lack the cell machinery and enzymes necessary for adenosine tri-phosphate (ATP) production (Beatty *et al.*, 1994a). Recent studies have revealed two potential ATP/ADP translocases and genes that may permit minimal amounts of ATP generation (Hatch, 1998). Chlamydial RBs multiply by binary fission within the inclusion before reorganising back into EBs at 24-48 hours p.i. (depending on species), resulting in an intracytoplasmic inclusion filled with EBs, RBs, and intermediate forms. EBs are released 48-72 hours later, depending on the species, by lysis or by fusion of the inclusion with the host cell membrane, allowing infection of further cells (Escalante-Ochoa *et al.*, 1998).

1.3 *Chlamydophila abortus* infections

C. abortus (formerly *Chlamydia psittaci* serotype 1) infects the placenta of sheep, causing a disease in sheep known as ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE) (Aitken, 2000b; Longbottom & Coulter, 2003). In Northern Europe it is the most common infectious cause of abortion in lowland flocks intensively managed at lambing time, and has a major economic impact on agricultural industries worldwide. Although there are no recent figures on the economic impact of the disease, losses have been previously estimated at £15-20

million per annum in the UK (Wood, 1992). The organism frequently causes a similar disease in goats, and also in cattle, pigs and horses, but such infections are thought to be less common.

1.4 Transmission and prevalence of *C. abortus* infection

The main routes of transmission of *C. abortus* are through ingestion of organisms shed in vaginal fluids and placental membranes at the time of abortion or lambing, or through inhalation of aerosols from the environment. EBs can remain viable in the external environment for several days, or possibly for several months depending on environmental conditions (Aitken *et al.*, 1990). This is due to the rigidity of the cell envelope, which is both osmotically stable and poorly permeable. There is some evidence of venereal transmission (Papp & Shewen, 1996b), but this is unlikely to contribute much to the epidemiology of EAE because of UK management practices, where there is little contact between ewes and rams during the lambing season (Appleyard *et al.*, 1985). Another potential route of transmission is through direct infection of the foetus via the placenta, although it is unclear what contribution this might make to the epidemiology of EAE (Buxton *et al.*, 1990).

An initial outbreak within a flock that may give rise to only a few abortions, can lead to over 30% of the ewes aborting or giving birth to stillborn or weak offspring in the following year (Aitken, 2000b). In subsequent lambing seasons the incidence of abortion is likely to remain at 5-10% if affected animals are left untreated. There was a resurgence of OEA in south east Scotland in the late 1970s (Linklater & Dyson, 1979), as a result of intensive management, increased sheep movement and vaccine breakdown, and this led to the spread of disease to other areas of Britain.

1.5 Clinical symptoms associated with *C. abortus* infections

Infection of pregnant ewes with *C. abortus* up to 5-6 weeks prior to parturition leads to clinical disease, and results in either abortion in the final 2-3 weeks of gestation, or the birth of stillborn or weak lambs that frequently die in the first few days of their

life (Aitken *et al.*, 1990). Infection of ewes within the last 5-6 weeks of pregnancy often leads to the development of a latent infection, where ewes appear to be uninfected until the next lambing season (Aitken *et al.*, 1990). Similarly, surviving lambs born to infected mothers may be affected by enzootic abortion in their first pregnancy (Wilsmore *et al.*, 1990b). Although, there are generally no premonitory indications of the impending abortions, vaginal discharges can be observed for up to 48 hours prior to lamb loss. These discharges can continue for 2-3 weeks, adding to the environmental spread of infection (Nietfeld, 2001).

In contrast to *Toxoplasma gondii* infections in pregnant sheep (Blewett & Watson, 1983), the timing of infection with *C. abortus,* whether early or mid-gestation, does not influence the pattern and progress of disease (Buxton *et al.*, 1990). Pathological changes are not detected in the placenta until day 90 of pregnancy irrespective of the timing of infection with *C. abortus* (Buxton *et al.*, 1990). Aborted placental tissue is thickened and necrotic, with inflammatory exudate present.

Experimental infection of ewes may cause a febrile response and enlargement of lymph nodes. Papp *et al.* (Papp *et al.*, 1993) suggest that *Chlamydophila* first enters the draining lymph node then spreads to the circulatory system and other tissues. Persistent infections may be present in lymph nodes, as a result of limited multiplication of the organism in infected lymph nodes. However, the organism may be present in a form (intermediate) undetectable by culture or immunohistochemistry (**Section 1.7**).

Infection of foetuses can occur before day 90 of gestation, as shown by culture of *C. abortus* from a foetus on day 75 of gestation, and detection of antibody in foetuses at both 75 and 95 days gestation (Buxton *et al.*, 1990). Sheep possess a non-invasive placenta, as described in **Section 1.9.1**, in which antibody cannot pass from mother to foetus, the detection of antibody suggesting direct infection of foetuses from the infected ewes. Later in pregnancy, there was a significant increase in immunoglobulin (Ig) M and IgG-positive cells in spleen and lymph nodes of foetuses from infected ewes (Buxton *et al.*, 1990) demonstrating the presence of an early

humoral response in the foetus, which is similar to that observed in *T. gondii* infection of pregnant ewes (Buxton & Finlayson, 1986). Further evidence of a foetal immune response was demonstrated by T and B cell stimulation in the foetal lymph nodes.

1.6 Immunity to chlamydial abortion

Ewes develop protective immunity following abortion, that prevents *C. abortus*-induced abortions in subsequent pregnancies (Wilsmore *et al.*, 1990b). Most ewes are seldom clinically affected following abortion and remain fertile. However, immunity is not necessarily sterile, as some ewes have chronically infected reproductive tracts and can continue to shed infectious organisms seasonally for up to three years, increasing the spread of disease to other sheep within the flock (Papp *et al.*, 1994). A small percentage of aborted ewes may develop secondary infections such as metritis (Papp *et al.*, 1993).

Papp *et al.* (1998) have demonstrated that ewes that had aborted in year one can have two subsequent successful pregnancies (years two and three) with the production of live, healthy offspring. They infected four ewes in the first pregnancy only and the other four ewes were infected in all 3 pregnancies. Papp *et al.* (Papp *et al.*, 1998) detected *C. abortus* by immunocytology in vaginal and cervical swabs, which were collected during the oestrus cycle, from the 8 chronically infected ewes, with no differences detected between those infected once or three times. Interestingly, higher numbers of organisms were present at the time of oestrus than at other sample times in all sheep (Papp *et al.*, 1998), which will lead to further spread of *C. abortus.* Obviously identification and removal/segregation of these ewes is a central control strategy (see **Section 1.12**). High levels of antibody responses were detected post-abortion, suggesting continued exposure to the organism despite only detecting it near oestrus (Papp *et al.*, 1994). Protective immunity is predominantly a T helper 1 (Th1) type response, as discussed in depth in **Section 1.11**.

1.7 Persistent chlamydial infections

Abortion due to *C. abortus* contaminates the surrounding environment and infects susceptible animals that come into contact with infected tissues/aerosols (McEwan, 1951). EAE then develops in subsequent pregnancies of ewes that are infected in a non-pregnant state. EAE also develops in the subsequent pregnancies of ewes that are infected late in the gestation period (in the last 5 or 6 weeks of pregnancy). *C. abortus* must therefore have the ability to establish a persistent infection that does not stimulate protective immunity at the time of infection. It has been suggested that *C. abortus* may reside in lymphoid organs during the latent phase of infection (Papp *et al.*, 1993).

Persistent, subclinical or latent states can be maintained for several months following infection of non-pregnant ewes, probably under control of the proinflammatory cytokine interferon-gamma (IFN-γ; see **Section 1.11.2.3**) (Brown & Entrican, 1996), before the onset of pregnancy triggers recrudescence of infection (McCafferty, 1990). During the subsequent pregnancy an unknown signal leads to stimulation and multiplication of the organism, and disease symptoms are manifested. However, *C. abortus* is undetectable by any means in latently infected ewes (Jones *et al.*, 1995), and organisms are not identified until day 90 of the subsequent pregnancy. *C. abortus* must be reactivated in some way in this pregnancy, resulting in clinical symptoms in the placenta, and ultimately abortion. A combination of hormonal changes and immunosuppressive factors associated with pregnancy are likely to lead to this recrudescence of *C. abortus* and abortion.

C. abortus is auxotrophic for the amino acid, tryptophan. Indolamine 2,3-dioxygenase (IDO) expression induced by IFN-γ breaks down L-tryptophan and depletes intracellular tryptophan pools (Byrne *et al.*, 1986). IFN-γ binds to target molecules (receptors) on the surface of epithelial cells and it has been suggested that IFN-γ inhibits the growth of *C. abortus* by inducing the production of IDO, which reduces the availability of tryptophan. This led to the production of persistent chlamydial forms that could be reactivated by the removal of IFN-γ or the addition of

excess tryptophan (Beatty *et al.,* 1994b). The depletion of tryptophan may lead to competition between host cells and *Chlamydia* for the essential amino acid, and result in chlamydiostasis (Graham *et al.,* 1995).

Trophoblast cells, the main target of *C. abortus,* are reported to constitutively express IDO and degrade tryptophan (Entrican *et al.*, 2004). Tryptophan catabolism appears to be an important mechanism for regulation of inflammatory responses during pregnancy, resulting in T cell tolerance and survival of the semi-allogeneic foetus. IFN-γ production decreases during pregnancy, which may explain the lack of control of infection and the reactivation of a latent *Chlamydophila* infection. The anti-inflammatory cytokines, interleukin (IL)-4 and IL-10, down-regulate IFN-γ during pregnancy in mice (Krishnan *et al.*, 1996). This has yet to be proven in sheep but if it is the case, down-regulation of IFN-γ may lead to chlamydiae being reactivated from their latent state, allowing colonisation of the placenta and disruption of the maternal-foetal junction, resulting in abortion.

Persistent or latent infections may develop as a result of an inadequate or sub-optimal T helper type 1 (Th1) response leading to a delay in the clearance of the pathogen and the production of a chronic response resulting in tissue damage (Yang & Brunham, 1999). For example, IL-10 (a Th2 cytokine) knockout (KO) mice exhibited strong Th1 and delayed-type hypersensitivity (DTH) responses that resolved pulmonary experimental infection without tissue damage (Yang *et al.*, 1996), whereas IFN-γ (a Th1 cytokine) KO mice exhibited poor Th1 and DTH responses and were unable to clear the infection (Wang *et al.*, 1999). Control of *C. abortus* in the persistent state is therefore linked to the balance of IFN-γ and the availability of tryptophan during pregnancy (Brown *et al.*, 2001).

1.8 Diagnosis of *C. abortus* infection in sheep

The first sign of *C. abortus* infection in a flock is generally abortion in the final 2-3 weeks of gestation, with the production of dead lambs and necrotic placental tissue. Earlier diagnosis of *C. abortus* infections in ewes is required before abortion occurs

to allow appropriate control measures to be made. In conjunction with this, infected and potentially infected ewes need to be treated to limit or prevent further spread of infection.

The presence of EBs in smears of potentially infected tissue samples or swabs from potentially infected ewes are detected by staining them with modified Ziehl-Neelsen (Aitken & Longbottom, 2004) *C. abortus* can also be isolated from infected placental cotyledons and vaginal swabs by culture in embryonated hens' eggs or in cell culture (Aitken & Longbottom, 2004). After growth, chlamydial inclusions can be visualised by staining either smears of yolk sac grown material or infected cell monolayers with Giemsa (Aitken & Longbottom, 2004).

Polymerase chain reaction (PCR) on samples prepared from infected cotyledons and swabs is an option if culture is not available (Aitken, 2000b; Amin, 2003b; Hartley *et al.*, 2001; Herring, 1991). However there is a possibility of false negative results due to the presence of inhibitors in the tissues (Caul & Sillis, 1998). PCR offers the advantage of distinguishing between *C. pecorum,* which is commonly found in faeces and can therefore contaminate diagnostic samples, and *C. abortus* infection by restriction fragment length polymorphism (RFLP) analysis of amplified PCR products (Anderson *et al.*, 1996; Denamur *et al.*, 1991; Everett *et al.*, 1999b). New PCR-tests are being developed, based on *C. abortus*-specific gene sequences to increase specificity and sensitivity.

Following abortion, maternal antibodies are produced in response to *C. abortus* infection (ewes seroconvert), coinciding with the development of protective immunity (**Section 1.11**), therefore serological diagnosis is only useful retrospectively (Amin, 2003a). Serological diagnosis of EAE has been mainly based on the complement fixation test (CFT). However the CFT is neither particularly sensitive nor specific since antibodies to other chlamydial species and enterobacterial pathogens may be detected as a result of antigenic cross-reactivity (Rodolakis *et al.*, 1998). The CFT was first developed and used in the 1950s (Stamp *et al.*, 1952), detecting antibodies to the genus-specific LPS antigen, which possesses several

epitopes, including a surface-exposed, immunoaccessible, genus-specific epitope. The broad specificity and the low sensitivity of the test have led to the development of other diagnostic techniques such as enzyme-linked immunosorbent assays (ELISA) and the afore-mentioned PCR. An optimal balance between sensitivity and specificity is required, due to the cross-reactivity with *C. pecorum* and lack of clinical signs associated with *C. abortus* infections.

A wide range of *C. abortus*-specific ELISAs have been produced, based on solublised proteins (Anderson *et al.*, 1995) or monoclonal antibodies (mAbs), which recognise specific MOMP regions (Salti-Montesanto *et al.*, 1997). ELISAs have also been developed based on recombinant protein fragments of the polymorphic outer membrane protein (POMP) family (Buendia *et al.*, 2001; Longbottom *et al.*, 2001). One example is an indirect ELISA based on a recombinant protein fragment of POMP91B, which was more sensitive and specific than the CFT (Longbottom *et al.*, 2001). The polymorphic membrane protein (*pmp*) gene family code for a cluster of surface-exposed immunogenic proteins, which are genus-specific (Longbottom *et al.*, 1996; Souriau *et al.*, 1994). They have been suggested as possible serodiagnostic candidates, due to their strong reactivity in immunoblotting experiments with sera from ewes infected with *C. abortus* (Cevenini *et al.*, 1991; Longbottom *et al.*, 1996) but not with sera from ewes infected with *C. pecorum* (Layachi *et al.*, 1993; Souriau *et al.*, 1994).

1.9 Immunopathogenesis of *C. abortus* infection

One of the most fascinating aspects of *C. abortus* infection is the lack of any pathology until after day 90 of gestation (a normal gestation period is approximately 143 days), irrespective of the timing of infection (Buxton *et al.*, 1990). Following an initial infection, it is thought that the organism establishes first in the tonsil, or by passing through the mucosa of the abomasum and small intestine, from where it is disseminated by blood and lymph to other organs (Jones & Anderson, 1988).

1.9.1 Placentation

Epitheliochorial, syndesmochorial, endotheliochorial and hemochorial placental types have been distinguished, denominating the layers between the foetal trophoblast and the maternal endometrial surface (Benirschke, 2002). **Table 1.2** shows the characteristic features of human, mouse and sheep placentation, followed by a description of ovine and murine placentation in **Sections 1.9.2** and **1.9.5**, respectively.

Table 1.2 Placenta classifications

Order	Species	Placental Form	Maternal-Foetal Interdigitation	Maternal-Foetal Separating Membrane
Primates	Ape, Humans	Discoid	Villi	Hemochorial
Rodentia	Rat, mouse	Discoid	Labyrinth	Hemotrichorial
Artiodactyla	Sheep	Cotyledonary	Villi	Epithelio- and Syndesmochorial

1.9.2 Ovine placentation

Ruminant placentation is cotyledonary, non-deciduate and synepitheliochorial (Entrican, 2002) (**Table 1.2**). The association between maternal and foetal tissues of the ovine placenta is less intimate in comparison to other types of placentation such as the single layer of contact between maternal and foetal vascular systems that occurs in the discoid hemochorial placenta of mice and humans (Engelhardt & King, 1996).

The uterine placenta of sheep is modified by invasion and fusion of binucleate cells, and is therefore generally referred to as synepitheliochorial. Cotyledons on the foetal side of the placenta are associated with rounded elevations of the uterine endometrium called caruncles and this unit is called a placentome (Studdert, 1968; Longbottom & Coulter, 2003). Caruncles are round thickenings in the uterine mucosa arising from proliferation of subepithelial connective tissue and are the only

site within the uterus to form attachments with foetal membranes. Contact between the foetal chorion and maternal epithelium is maintained by microvillous interdigitation in the placentome, through penetration of chorionic villi into maternal crypts (Engelhardt & King, 1996). Approximately 75 to 125 placentomes are present in the placenta of a pregnant ewe (Benirschke, 2002). Haematomas form at about day 60 of pregnancy, as a result of normal physiological invasion of the caruncular stroma by chrorionic villi coinciding with haemorrhage from blood vessels (Studdert, 1968).

A large number of binucleate cells are present within the ovine placenta. Binucleate cells arise early as part of the foetal trophoblast from cells that fail to undergo cytokinesis following nuclear division in the foetal trophectodermal epithelium (Wooding, 1982). Binucleate cells migrate across the maternal-foetal junction (Wooding, 1982) and then invade and fuse with uterine epithelial cells or caruncular epithelial cells to form chimeric trinucleate cells or other binucleate cells (Engelhardt & King, 1996). Binucleate cells appear to play a central role in the formation of structures and secretions at the maternal-foetal interface, which may play a crucial role in establishment and maintenance of pregnancy (Wooding, 1982). One of the major cellular functions of binucleate cells is the secretion of the placental hormone, lactogen. Ovine placental lactogen is secreted in large quantities from about day 50 of gestation and foetuses also accumulate high levels. Progesterone and other progestins, oestrogens and placental lactogen are the major hormones in the placenta (**Section 1.10**).

1.9.3 Infection of the ovine placenta with *C. abortus*

Maternal haematomas develop in ovine placentas at the maternal-foetal interface in the hilus of the placentome on day 60 of gestation (Studdert, 1968; Longbottom & Coulter, 2003). This results from leakage of blood at the septal tips of the maternal caruncle, and probably corresponds to the time that chlamydial organisms pass from mother to foetus. Despite the development of these haematomas and their possible role in infection, pathological changes, as a result of *C. abortus* infection, are not

evident until day 90. There may not be sufficient release of organisms from the maternal blood to induce any changes prior to this time point, or the placenta may be resistant to infection until day 90 of gestation. Hormonal and immunological variations linked to the pregnancy status are likely to play a role in *C. abortus* infection and abortion.

Initial placental lesions are a consequence of replication of the organism in inclusion bodies in the trophoblast cells of the chorionic epithelium (Papp *et al.*, 1993). Following establishment of infection in the foetal trophoblastic epithelial cells, infection spreads out into the intercotyledonary regions of the chorion, resulting in epithelial damage, oedema and inflammation. This inflammation is a result of infiltration by neutrophils, macrophages, lymphocytes and plasma cells. Not all of the placentomes become infected, and the level of inflammation and necrosis in the cotyledons and membranes is variable (Buxton *et al.*, 2002). Necrosis and inflammation within the endometrial epithelium indicate the further spread of infection. Ultimately, the damage caused by infection in the placentomes compromises the maternal-foetal exchange of nutrients and oxygen, which contributes to foetal death and abortion (Buxton *et al.*, 2002). Foetal infection is also possible and is characterised by focal necrosis in the liver. Small foci of necrosis may also be found in the lung, spleen, brain and lymph nodes (Buxton *et al.*, 2002), implying that the maternal-foetal interface is disrupted in some way, allowing the organism to reach the foetus (Buxton *et al.*, 1990).

1.9.4 Use of animal models

Animal models have been used for many years to study many aspects of disease and immunity. Mouse models have proved exceptionally useful in terms of manipulation of immune systems to investigate functions of particular cells. The best defined model of immunity to *Chlamydia* is infection of the murine genital tract with the mouse pneumonitis (MoPn) strain (*C. muridarum*) (Cain & Rank, 1995; Qiu *et al.*, 2004; Yang *et al.*, 1996). Animal models have also allowed a faster and cheaper

method of screening vaccine studies (Rekiki *et al.*, 2003; Hechard *et al.*, 2003a; Hechard *et al.*, 2003b).

Murine models have been used to study *C. trachomatis* genital infections during pregnancy (Pal *et al.*, 1999). Such animal models have investigated the role of *C. trachomatis* on the outcome of pregnancy and on the mechanisms that may be involved in the pathogenesis of the disease. The effects of *C. trachomatis* on pregnancy remain controversial within animal models, the outcome depending on factors such as the infecting inoculum, the time of gestation, and the susceptibility of the host. Pal *et al.* (1999) described a wide range of clinical manifestations, from asymptomatic infection to abortion, produced in *C. trachomatis*-infected mice. There are obvious differences between humans and mice, thus there are limitations as with any model system. However, mice have been successfully used to characterise the effects of *Chlamydia* on pregnancy. Studies have shown that the host response varies between mouse strains, and that this has an influence on the outcome of infection (Pal *et al.*, 2001; Ramsey *et al.*, 2000).

1.9.5 Mouse placentation

The hemochorial placenta of rodents is the most invasive type of placenta and is similar to that of humans, in contrast to the non-invasive ruminant placenta (Engelhardt & King, 1996). The majority of mouse placentas are separated from each other, although there are exceptions. Mouse placentas are flat, spherical structures and are smooth on the foetal side, where they are covered by cuboidal amitotic epithelium (Benirschke, 2002).

Mouse placentae are composed of folded villous tissues with very thin foetal capillaries covered by the trophoblast cell layer, termed the placental labyrinth, and these are immersed in the maternal sinusoidal blood (Gude, 1982; Ward & Devor-Henneman, 2000), as shown in **Figure 1.2**. This region constitutes the major site of maternal/foetal exchange, enhanced by maternal blood directly bathing the surface of the trophoblast cells i.e. hemochorial (Benirschke, 2002).

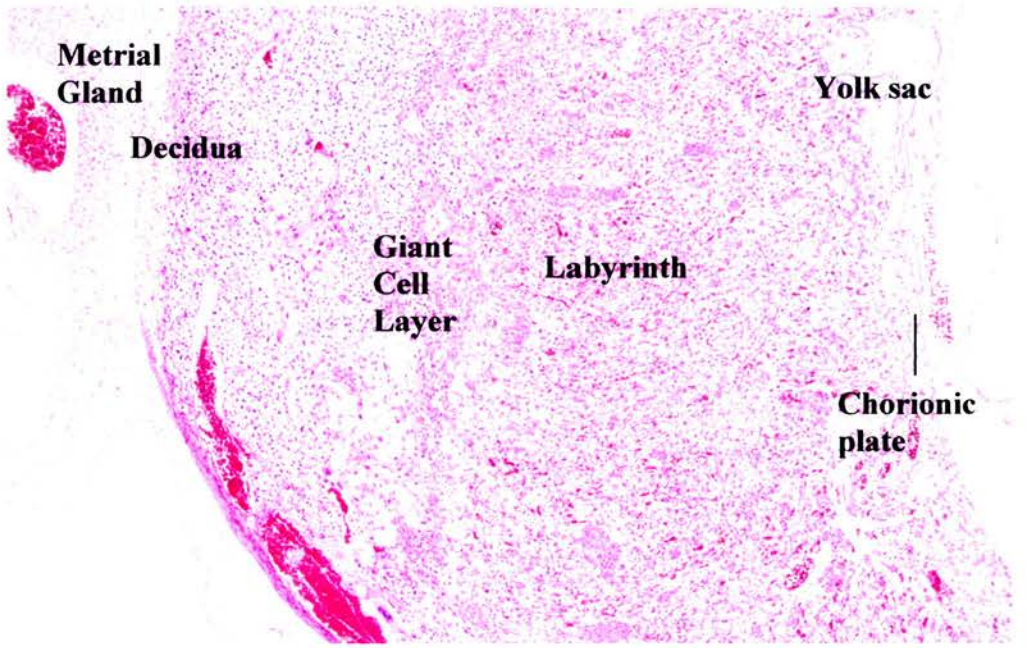


Figure 1.2 Subsection of murine placenta illustrating the main features of a mouse placenta. It is composed of separate cellular layers: the maternal decidua, the spongiotrophoblast (near the giant cells), the giant cell layer and the labyrinth bathed in maternal blood. The chorionic plate is the area closest to the foetus, where the yolk sac is inserted.

The foetal labyrinth is the major portion of the placental disk, as shown in **Figure 1.2,** and has very thin foetal capillaries that are supported by a tiny amount of connective tissue. This labyrinth area increases to over half of the total placental volume between days 12 and 17 post-coitum (dpc). Embryonic trophoblast cells and embryonic blood vessels lined by embryonic endothelium are present in the foetal labyrinth. Nucleated erythrocytes are present within these vessels and maternal mature erythrocytes are in the spaces lined by embryonic labyrinth trophoblasts in this area (Ward & Devor-Henneman, 2000). The umbilical cord originates in the chorionic plate, which is situated closest to the foetus, and foetal capillaries derived from umbilical blood vessels branch and interconnect with sinuses containing maternal blood, surrounded by trophoblast cells in the labyrinth (Zuckermann & Head, 1986). There are three different types of trophoblast cells found in the mouse placenta: giant cells, spongiotrophoblast and labyrinthine trophoblast (**Figure 1.2**). Other cells present include foetal and maternal blood cells, maternal decidual cells,

foetal endothelial and mesenchymal cells and macrophages (Zuckermann & Head, 1986). The trophoblast cells are involved in physiological exchange and hormonal production and the visceral yolk sac placenta acts as the major site of maternal/foetal exchange (Ward & Devor-Henneman, 2000).

Spongiotrophoblast cells produce steroid hormones (oestrogen, progesterone, lactogen and chorionic gonadotrophin), and other factors used in maternal-foetal communication, as maternal blood vessels run through this cell layer. These cells have also been shown to be phagocytic for maternal red blood cells, which may be a mechanism for iron transport to the foetus (Ward & Devor-Henneman, 2000). There are also endometrial glands, fibrinoid and trophoblast cells present. Another layer of trophoblast-derived cells between the spongiotrophoblast and the decidua is present, which contains giant cells. Giant cells are the primary sites of attachment to the endometrium, surrounding the entire membranes, and composing the trophospongium. Giant cells are found at the interface and connect the foetal placenta to the maternal decidua. The conceptus is separated from the maternal tissue by a continuous layer of giant cells by day 8 dpc. Giant cells of varying sizes are observed during the gestation period as a result of the cells discontinuing their mitotic activity and gradually increasing in size (Muntener & Hsu, 1977b). Giant cells are no longer formed after day 10 dpc therefore as the placenta grows, the layer of giant cells becomes narrower. These cells acquire endocrine function, immune functions and act as a source of cytokine production (Cross, 2001).

The maternal portion of the placenta is termed the decidua, containing large polygonal cells, which are rich in glycogen and lipids, and maternal blood cells within blood vessels. Irregular masses of an eosinophilic, homogenous substance called fibrinoid are present in new placenta, which increases with the age of the placenta, between the giant cell zone and the decidua (Muntener & Hsu, 1977a). Immunoglobulins cross this barrier from murine mother to foetus, contrasting with the lack of Ig transfer between the ewe and foetus in the ovine placenta (Field & Barnes, 1992; Ward & Devor-Henneman, 2000).

Granulated metrial gland (GMG) cells are found in the uterine stroma, their distribution depending on the development stage of the placenta (Stewart & Peel, 1978). They are thought to be associated with the development of the foetus (Peel & Bulmer, 1977). The position of the granulated cells in the implantation site and their association with maternal vessels, allows easy access to the foetal placenta. It has also been suggested that metrial gland cells have associations other than the implantation site as these cells have been detected in blood vessels elsewhere, suggesting that they have a function at all stages throughout pregnancy.

1.9.6 Mouse models of *C. abortus* infection

Experimental infection of pregnant mice with *C. abortus* results in infection of the placenta and abortion occurs as observed in ovine infections (Buendia *et al.*, 1998; Buzoni-Gatel & Rodolakis, 1983). Obviously there are distinct differences in placenta structure and lengths of pregnancy in sheep (epitheliochorial, 143 days) and mice (hemochorial, 21 days), yet the same outcome results from infection with *C. abortus* in the two distinct animal groups.

The first *C. abortus* pregnant mouse model, developed by Buzoni-Gatel and Rodolakis (1983) was shown to differentiate abortion and intestinal chlamydiae isolates of ovine origin following footpad inoculation. In this study, the abortion isolate (AB7) was suggested to be more virulent than the intestinal isolate, demonstrated by its ability to colonise the spleen and popliteal lymph node, and this has since been demonstrated in the ovine host (Huang *et al.*, 1990a). This model has been used to compare virulence of chlamydial strains (Rodolakis *et al.*, 1989), as well as being used in pathological (Sanchez *et al.,* 1996) and protection studies (Buzoni-Gatel & Rodolakis, 1983; de Sa *et al.*, 1995).

Buendia *et al.* (Buendia *et al.*, 1998) described an increase in chlamydial antigen and infectious foci in the liver of pregnant mice in comparison to non-pregnant mice following infection with *C. abortus*. Initial studies investigated the anatomical progression of *C. abortus* within the placenta, demonstrating that decidual cells at the

boundary of the maternal-foetal interface were the first cells to be infected. Neutrophil infiltration and necrosis at the materno-foetal interface are characteristic of chlamydial infections in sheep (Buxton *et al.*, 2001; Buxton *et al.*, 2002; Navarro *et al.*, 2004) and this has also been demonstrated in the mouse model (Buendia *et al.*, 1998). Infection was observed to spread to the layer of giant cells, with complete colonisation of the maternal placenta occurring after day 15 of pregnancy. Foetal labyrinth trophoblast cells were infected from day 18 of gestation onwards, a similar progression of infection from the maternal to the foetal side of the placenta to that observed during ovine infection. Neutrophil infiltration is characteristic of both mouse and sheep infections, which is likely to be one of the pathogenic mechanisms that leads to abortion (Buendia *et al.*, 1998).

Granulated metrial gland (GMG) cells are the most numerous lymphoid cells in the uteroplacental area in murine pregnancy (Sanchez *et al.*, 1996). These cells contain granules with cytotoxic molecules (perforins and serine esterases), which are a suggested defence mechanism against intracellular pathogens. *C. abortus* did not invade GMG cells until day 15 of gestation, which coincides with their apparent loss of function. Although there are differences between sheep and mouse placentae immune cells found and the type of immune response, there are cells in the endometrial tissues of sheep ($\gamma\delta$ $TCR^{+}CD8^{+}$) analogous to the GMG cells of pregnant mice (Buendia *et al.*, 1998).

Since *C. abortus* targets the placenta, it has been speculated that the susceptibility of GMG cells to infection could affect the degree of infection that is established (Sanchez *et al.*, 1996). Trophoblast cells are also suggested to play a role in the maternal immune response to bacterial infection at the utero-placental interface (Guleria & Pollard, 2000). This has been demonstrated by trophoblast responsiveness to colony-stimulating factor (CSF)-1, which is synthesised in high concentrations by the uterine epithelium during pregnancy. In *Listeria monocytogenes* mouse infections, CSF-1 was required to recruit neutrophils to the site of infection in the decidua basalis, and in its absence infection remained uncontrolled (Guleria & Pollard, 2000). Therefore, in mice, the trophoblast may act as a pregnancy-specific

component of the innate immune system, which may have relevance in *C. abortus* infections given the influx of neutrophils to sites of infections (Buendia *et al.*, 1998).

1.10 Hormonal influences on *C. abortus* infections

Hormones involved in pregnancy are likely to play a crucial role in inducing abortion following *C. abortus* infection, and progesterone, 17β-oestradiol and prostaglandin E_2 (PGE_2) are all central to pregnancy (Longbottom & Coulter, 2003; Entrican *et al.*, 2001). Progesterone is necessary for the maintenance of normal pregnancy and is produced in chorionic epithelial cells in the latter part of pregnancy. It then interacts with oestradiol and PGE_2 to control the onset of parturition. Progesterone has been referred to as the 'hormone of pregnancy' (Ragusa *et al.*, 2004) because it is involved in preparing the endometrium for implantation and facilitating endometrium development and it is also critical for the survival of a foetus. In addition, this hormone prevents the rejection of the foetal semi-allograft.

Progesterone levels decrease in chlamydial infected placentas, whereas the concentrations of oestradiol increase in the amniotic and peripheral plasma, and PGE_2 concentrations increase in the amniotic and allantoic fluids (Leaver *et al.*, 1987; Leaver *et al.*, 1989). The changes in hormonal levels during pregnancy may increase susceptibility to infection, and promote placental infection at about day 90 of gestation (Buxton *et al.*, 1990) and it has been suggested that this hormone imbalance is an underlying cause of abortion (Buxton & Henderson, 1999). Indeed, a study of *C. trachomatis* infection in rats demonstrated that progesterone enhanced susceptibility to disease while oestradiol decreased susceptibility (Kaushic *et al.*, 2000). Since multiplication of chlamydial organisms takes place in the foetal chorionic epithelial cells, the main source of progesterone in the latter stages of pregnancy, damage to this area is likely to play a role in altering the hormonal balance, which is necessary for the maintenance of normal pregnancy, resulting in premature labour.

Hormones also affect T cell reactivity and cytokine production. For example, progesterone is involved in the development of T helper type 2 (Th2) cells that produce IL-4 and IL-5, 17β-oestradiol enhances IL-10 and IFN-γ secretion by antigen-stimulated clones and PGE_2 biases the cytokine production of dendritic cells towards IL-10, reducing IL-12 production (Entrican, 2002). The shift in immune response toward a Th2 pattern may benefit the foetus, whereas development of proinflammatory response (Th1) may be harmful (Ragusa *et al.,* 2004). Thus, in pregnant animals, the inflammatory response due to the invading organisms in association with changes in hormone levels alters the placental environment and endangers the developing foetus (Buxton *et al.*, 2002; Entrican *et al.*, 2001).

1.11 Immune responses to *C. abortus*

Both cell-mediated and humoral immune responses are involved in immunity to chlamydial infections (Buzoni-Gatel *et al.*, 1987; Williams *et al.*, 1984). Knockout mouse experiments have been used to demonstrate the role of B cells in *C. trachomatis* infections, with B cell-deficient mice being more susceptible to re-infection than wildtype (WT) mice (Williams *et al.*, 1997). However, the cellular response is more efficient in decreasing subsequent infection in this model, implying that the cell mediated immune response plays a more significant role in the resolution of chlamydial infection than the humoral response. Immunity to *C. abortus* infections has been demonstrated by the transfer of primed splenic T cells and by the transfer of sera from immune mice (Buzoni-Gatel *et al.,* 1987), highlighting the importance of both cell mediated immunity and humoral immunity, although as is well known, the former plays a more dominant role in the resolution of infections. $CD4^+$ T cells are the predominant responding cell type in murine *C. trachomatis* infections, while it has been suggested that $CD8^+$ T cells assume this role in the host defense against murine *C. abortus* infections (de Oca *et al.*, 2000a).

Table 1.3 shows a summary of the major immune cells and their general role in controlling infections. The following sections then describe the role of various cell types in the specific control of *Chlamydia* infections, focusing on *C. abortus* infections.

Table 1.3 Overview of immune cell types and functions

Cell	Classification	Stimuli	Function
Monocyte	Phagocyte	GM-CSF; IL-4; microbial components; macrophages; complement	Cytokine release; differentiation into macrophages and dendritic cells
Macrophage (in liver tissue termed Kupffer cells)	Antigen presenting cell	IFN-γ; microbial components; IL-4 & IL-13; or IL-10	Phagocytosis, microbial killing, inflammation
Dendritic cell	Antigen presenting cell	Microbial components; IFN-γ; TNF	Host defence; phagocytosis; Ag presentation; cytokine release; direction of adaptive immunity
Neutrophil	Granulocyte; phagocyte	Chemokines; microbial components; complement	Phagocytosis and microbial killing; cytokine release; inflammatory-mediator release
Eosinophil	Granulocyte; antigen presenting cell	IL-5; eotaxin; IgA; IgE	Allergy-mediating cell; helminth defence; cytokine release
Basophil	Granulocyte	IL-3; IgE-antigen complexes; IgA (if primed)	Allergy-mediating cell; immunity to nematodes; inflammatory mediator release; secretion of IL-4 and IL-13
Mast cell	Granulocyte	IgE-antigen complexes; neuropeptides; complement; bacteria; cytokines	Allergy-mediating cell; inflammatory cytokine
B cell	Lymphocyte	Foreign antigens; self-antigens; IL-4; IL-10; IFN-γ; IL-2	Mediator of humoral immunity; antibody production; antigen presentation; lymph tissue development
T cell	Lymphocyte	Antigen presentation; co-stimulatory receptors; IL-4 ; IL-12	Direct killing of targeted cells; stimulation of cell-mediated and humoral immunity (see later for more details on subsets)
Natural killer cell	Lymphocyte	Antibody; lack of target cell MHC I; IFN-α; IFN-β; IL-12; TNF-α	Host defence against viral infection and cancer; cytolytic activity; cytokine release; primary role in innate immunity

1.11.1 Humoral response

It is well documented that ewes seroconvert post-abortion (Aitken, 2000a; Longbottom *et al.*, 2001; Longbottom *et al.*, 2002) (described in **Section 1.8**), although the contributory role of antibody in resolving primary infections remains unclear (Longbottom and Coulter, 2003). Antibody has also been readily detected both in serum and secretions from animals infected in the genital tract with *C. trachomatis*, although the role of antibody in primary infections is contrasting in the guinea pig and mouse models of chlamydial infection (Rank, 1999). A definitive role for the humoral response in the resolution of infection was confirmed in the guinea pig model (Rank *et al.*, 1979) whereas antibody was not necessary for the resolution of infection in mice (Ramsey *et al.*, 1988).

In contrast, B cells have been ascribed an important role in the early response to *C. abortus* infection in mice. B cell-deficient mice were more susceptible to *C. abortus* infection shown by increased mortality, significantly higher levels of infection and an exacerbated inflammatory response which led to thrombi and necrosis in infected tissues compared to WT mice (Buendia *et al.*, 2002a). However, Buendia *et al.* (2002a) demonstrated that, following re-infection after a low-level primary infection, B-cell deficient mice were protected from this secondary challenge and T cells were primed even in the absence of B cells, shown by an increased IFN-γ response. This was also demonstrated by Johansson and Lycke (Johansson & Lycke, 2001) and Morrison *et al.* (Morrison *et al.*, 2000) in a genital tract model infection with *C. trachomatis*. Morrison *et al.* (2000) reported the significant role of B cells in resistance to chlamydial reinfection in this *C. trachomatis* genital tract mouse model. Depletion of $CD4^+$ T cells in WT mice had a limited effect on resistance to re-infection whereas depletion of $CD4^+$ T cells in immune B cell-deficient mice resulted in mice being unable to resolve a secondary chlamydial infection, showing the importance of both $CD4^+$ T cells and B cells (Morrison *et al.*, 2000). It may be that antibodies act to enhance Th1 activation via FcR-mediated processes, such as the enhanced uptake and processing and presentation of antigens, in resistance to re-infection (Moore *et al.*, 2002, 2003). This also highlights the requirement of potential

protective vaccines to elicit both humoral and cell-mediated immune responses (Moore *et al.*, 2003).

Studies in Fc-receptor (FcR) KO mice have shown that antibodies have a major role in resistance to re-infection with *C. trachomatis* (Moore *et al.*, 2002), suggesting that FcR-dependent effector functions may represent the major mechanisms by which antibodies participate in control of re-infections. FcRs bind the Fc portion of immunoglobulin and function as important vehicles for antibodies to enhance the phagocytosis of pathogens, to initiate intracellular degradation and removal, and for antigen processing/presentation for immune elicitation. Binding of FcRs by antigen-antibody complexes can activate immune cells bearing the receptors, such as phagocytes, macrophages, polymorphonuclear neutrophils (PMNs), natural killer (NK) cells, eosinophils and mast cells (Moore *et al.,* 2002). FcR-mediated immune processes have also been examined *in vitro* and showed that specific anti-chlamydial antibodies augmented macrophage killing of infected cells by antibody-dependent cellular cytotoxicity (ADCC) and macrophage inhibition of growth of chlamydiae in co-cultures (Moore *et al.,* 2002).

Antibody responses in the ovine immune response to *C. abortus* comprise whole IgG and IgM responses in sera (Papp *et al.*, 1994) and IgG and IgA have also been detected in vaginal secretions from chronically infected ewes (Papp & Shewen, 1997). Subclasses of serum antibodies can be used as an indicator of type 1 or 2 T cell dominance (Finkelman *et al.*, 1990). In mice, the type 1 (Th1) cytokine IFN-γ enhances IgG2a production by B cells but inhibits IgG1 (Stevens *et al.*, 1988), therefore IgG2a is reflective of a type 1 T cell response (Snapper & Paul, 1987). Increased IgG2a production compared to IgG1 has been demonstrated in *C. abortus* infected mice (Del Rio *et al.*, 2000).

1.11.2 Cell-mediated response

1.11.2.1 Delayed type hypersensitivity

Early research on *C. abortus* immune responses demonstrated the importance of cell mediated immunity in resolving infection. Delayed type hypersensitivity (DTH) is important for resistance to EAE, and IFN-γ, which controls infection, has an important role in DTH. Delayed type hypersensitivity may therefore play a role as a direct effector mechanism in the generation of immunity as well as maintaining persistence in sheep. Ewes that have aborted have positive DTH reactions, compared to no DTH responses in non-pregnant infected ewes (Dawson *et al.*, 1986; Wilsmore *et al.*, 1986). Sheep with poor DTH responses following vaccination were more likely to abort post-challenge, suggesting DTH could be used as a measure of immunity.

1.11.2.2 The role of CD4 and CD8 T cells in *Chlamydia* infections

The induction and recruitment of specific T cells into the infected environment is necessary for controlling chlamydial infections (Igietseme *et al.*, 2002). Early studies of efferent lymph collected from infected sheep revealed that $CD4^+$ and $CD8^+$ T cell populations were present (Huang *et al.*, 1990a), and this occurs in both naive and immune sheep (Entrican *et al.*, 1998). The relative importance of both $CD4^+$ and $CD8^+$ T cells during primary infection has been in dispute for many years, although $CD4^+$ cells appear to be more dominant and have been shown to produce IFN-γ *in vitro* in response to challenge with chlamydial antigen (Byrne *et al.*, 1989). The role of IFN-γ in control of infections is discussed in more detail in **Section 1.11.3.1**.

On the other hand, Beatty and others (1994a,b) have suggested that *Chlamydia*-infected cells are lysed by specific cytotoxic T lymphocytes (CTLs; $CD8^+$ T cells). $CD8^+$ T cells could also be ascribed a cytotoxic role after recognition of *C. abortus* antigen complexed with major histocompatability complex (MHC) class I, and via production of the cytokine IFN-γ (Beatty *et al.*, 1994a,b). Although this evidence implies that $CD8^+$ T cells do not act in a cytotoxic manner, but act in a similar way to

$CD4^+$ T cells by enhancing IFN-γ expression (Buzoni-Gatel *et al.*, 1992). Infection of mice with other chlamydial species such as *C. trachomatis,* which causes human genital tract infection, leads to recruitment of both $CD4^+$ and $CD8^+$ lymphocyte populations to sites of infection in the uterus (Reddy *et al.*, 2004). Elimination of $CD4^+$ and $CD8^+$ T cells in MoPn infection significantly increased pathogen load in the lungs and decreased antigen-induced IFN-γ by both $CD4^+$ and $CD8^+$ T cells (Magee *et al.*, 1995). Depletion of $CD4^+$ cells led to a significant increase in mortality, whereas depletion of $CD8^+$ cells did not, although higher mortality was observed than in non-depleted mice. Irrespective of their roles, both T cell populations lead to the upregulation of cytokines, IFN-γ and tumour necrosis alpha (TNF-α), which are involved in immunity to *C. abortus* (Buxton *et al.* 2002; Byrne *et al.,* 1986; Entrican *et al.,* 2001).

Two responder phenotypes of $CD4^+$ T cells exist, defined by the different cytokine patterns that they express. Th1 cells, induced by infection with intracellular bacteria and viruses, secrete cytokines such as IFN-γ, IL-2, IL-12, and TNF-α. Th2 cells, normally induced in response to allergens and helminth parasites, secrete cytokines such as IL-4, IL-5, IL-10 and IL-13 (Mosmann & Coffman, 1989). Once a T cell response starts to develop along a Th1 or Th2 phenotype it becomes increasingly polarised. In sheep, *C. abortus* is controlled by a Th1 type immune response, with IFN-γ and TNF-α playing major roles (Buxton *et al.,* 2002; Entrican *et al.,* 2001). Ruminants may be simultaneously infected with gastrointestinal nematodes and *C. abortus* in the field. Buendia *et al.* (Buendia *et al.*, 2002b) conducted co-infection studies in mice with *C. abortus* and *Nippostrongylus brasiliensis*, a rodent gastrointestinal nematode used extensively in experimental models of parasite-induced Th2 responses (Finkelman *et al.*, 1997; Holland *et al.*, 2005) to determine the effect of a previously established immune response to parasite infection on the response to *C. abortus* infection. Mice that were previously infected with *N. brasiliensis* had an increased susceptibility to *C. abortus* infection, demonstrated by increased infection levels and lower IFN-γ production compared to mice infected with only *C. abortus*, suggesting that an already established Th2 response alters the

normal Th1 response induced in response to *C. abortus* infections in mice (Buendia *et al.*, 2002b).

1.11.3 Inflammatory response to *C. abortus* infections

1.11.3.1 Cytokine response

Two inflammatory cytokines, IFN-γ and TNF-α, which are produced in response to *C. abortus* infections, are thought to contribute to the pathology of EAE and also to threaten the maintenance of pregnancy in sheep (Entrican, 2002). Mononuclear cells expressing mRNA encoding TNF-α were shown to be abundant in infected placental tissues, particularly in the inflammatory exudates associated with the chorionic epithelium, although very few cells expressed mRNA encoding IFN-γ (Buxton *et al.*, 2002).

IFN-γ, produced by both $CD4^+$ and $CD8^+$ T cell subsets, is crucially involved in controlling chlamydial infections (Loomis & Starnbach, 2002; Rottenberg *et al.*, 2000). IFN-γ can activate the microbicidal activity of host macrophages and the macrophages can cause destruction of chlamydial-infected cells *in vitro*, suggesting its importance in host resistance (McCafferty *et al.*, 1994; Igietseme, 2002). It has been demonstrated that *C. abortus* multiplication can be restricted by IFN-γ in ovine cells *in vitro* (Graham *et al.*, 1995). IFN-γ is expressed in placental tissue during normal pregnancy, but it can be cytotoxic for human trophoblast cells at high concentrations (Yui *et al.*, 1994). Therefore, an increase in IFN-γ expression in response to chlamydial infection within placenta tissue may be detrimental to both the maternal and foetal host tissue as well as to the invading organisms. IFN-γ inhibits growth of *Chlamydia* both *in vitro* (Entrican *et al.*, 1998) and *in vivo* (Lampe *et al.*, 1998). It seems highly likely that the presence of IFN-γ controls the growth of *C. abortus* within host tissues, despite the low incidence of placental cells expressing IFN-γ mRNA, and simultaneously impairs foetal survival by impairing the function of placental trophoblast cells due to a decrease in tryptophan, which in turn damages placental cell functions and contributes to abortion. Macrophage production of

inflammatory mediators is triggered by IFN-γ and this causes fibroblast proliferation, thereby enhancing the synthesis of collagen (Beatty *et al.*, 1993), which is also likely to contribute to pathology in infected sheep. IFN-γ inhibition of multiplication results in inapparent persistent infections, and this is likely to also play a role in the immunopathogenesis associated with *Chlamydia* infections (Rottenberg *et al.*, 2002).

IFN-γ-gene KO mouse models have been developed, and alongside *in vitro* studies, have proved extremely useful in demonstrating the essential role of IFN-γ in controlling all chlamydial infections (Dalton *et al.*, 1993; Lampe *et al.*, 1998; Rottenberg *et al.*, 2000). Cotter *et al.* (Cotter *et al.*, 1997) demonstrated the importance of the role of IFN-γ in resolving *C. trachomatis* infection in the MoPn model system, using mice in which the gene for IFN-γ had been disrupted. Wildtype mice infected with *C. trachomatis* MoPn showed systemic dissemination of a small number of organisms to major organs from day 7-14 p.i. and the infection resolved within 3 weeks. However, the ability to resolve infection was impaired in IFN-γ KO mice, where a prolonged and more pronounced systemic dissemination of *Chlamydia* was observed, as demonstrated by significantly higher numbers of organisms cultured from spleen tissue (Cotter *et al.*, 1997).

TNF-α is thought to play a crucial role in immunity to *Chlamydia* through the activation of phagocytic cells that rapidly degrade infected cells or extracellular EBs (Igietseme *et al.*, 2002). A high concentration of TNF-α at the maternal-foetal interface is incompatible with pregnancy (Dealtry *et al.*, 2000) and TNF-α has been linked to spontaneous embryo loss in pregnant mice (Yui *et al.*, 1994). Therefore, TNF-α released in response to chlamydial infection (possibly to the LPS component) may contribute to the placental damage that leads to abortion as well as playing a role in controlling the disease (Buxton *et al.*, 2002).

IL-12 is important for initial clearance of bacteria, while IFN-γ is important for long-term resolution of infection (Kaushic *et al.*, 2000). The role of dendritic cells in immunity to chlamydial infection has been demonstrated through the production of IL-12. IL-12 promotes a Th1 type response, by acting on NK cells to stimulate the

production of IFN-γ (Lu & Zhong, 1999; Scheerlinck *et al.*, 1998; Tseng & Rank, 1998). However, IL-12 is not necessarily essential for controlling *C. abortus* infections as IL-12 KO mice mounted a protective Th1 immune response against the infection, by the production of both IFN-γ and IgG2a (Del Rio *et al.*, 2001). $CD4^+$ T cell infiltration was reduced in IL-12 KO mice compared to WT mice, although the number of $CD8^+$ cells was slightly higher, which is consistent with a role for $CD8^+$ T cells in the protective response leading to the production of IFN-γ (Del Rio *et al.*, 2001).

In summary, a CD4 Th1 immune response is thought to be the principal component of the protective response to chlamydial infection, with IFN-γ and TNF-α being major cytokines involved in the response (Buxton *et al.*, 2002; Zhong *et al.*, 2000). Both antibodies and $CD8^+$ T cell subsets are also major components of the protective immune response, the former as a memory response during re-infection, and the latter contributing via cytokine production, rather than by cytotoxicity, complementing the $CD4^+$ Th1 driven response (Igietseme *et al.*, 2002).

1.11.3.2 Role of PMNs in chlamydial infections

Pronounced neutrophil infiltration is observed in the placenta of mice infected with *C. abortus* and those of sheep infected under natural conditions (Buendia *et al.*, 1999; Buxton *et al.*, 1990; Wong *et al.*, 1985). PMNs have previously been reported to play a role in the resolution of *C. trachomatis* infections (Barteneva *et al.*, 1996), and neutrophil infiltration of the uterus is not unique to chlamydial infections; for example, *Brucella abortus* and *L. monocytogenes* infections also cause infiltration of PMNs in the murine placenta (Redline *et al.*, 1988; Tobias *et al.*, 1993). Neutrophils could be beneficial to the host by ingesting the organism and clearing the infection. Alternatively they could be detrimental by causing placental damage, inflammation and necrosis as a result of recruitment of other cell types, and thereby be involved in inducing abortion.

PMNs are suggested to play an essential role in the response to primary infection with *C. abortus* (Buendia *et al.*, 1999). Studies showed that depletion of PMNs resulted in earlier abortions and a higher mortality rate in mice infected with *C. abortus* (Buendia *et al.*, 1999). Depleted mice expressed widespread necrosis of the uteroplacental units in contrast to virtually no necrosis in non-depleted mice. Register *et al.* (Register *et al.*, 1986) and Yong *et al.* (Yong *et al.*, 1986) demonstrated the ability of PMNs to destroy *Chlamydia in vitro*. However, PMNs are suggested to have a limited role in the secondary response, demonstrated by no clinical signs in both WT and depleted mice upon secondary infection with *C. abortus* (de Oca *et al.*, 2000b). Neutrophils also appear to play an immunoregulatory role, by secreting cytokines such as TNF-α, IFN-γ and IL-12, which in turn activate a Th1 response that could lead to control of the infection (Ellis & Beaman, 2004).

1.12 Treatment and control methods

Current control methods for EAE are limited. Clinically affected and in contact pregnant animals are preferably treated with long-acting oxytetracycline, which when given at later stages of infection can prevent abortion (Aitken *et al.*, 1982; Rodolakis *et al.*, 1980). Oral tylosin is also frequently administered (Rodolakis *et al.*, 1980). Treatment of a whole flock in which ewes are aborting for the first time can reduce losses, as antibiotics inhibit chlamydial growth and limit additional placental damage (Aitken *et al.*, 1982). However, with these drugs alone, complete elimination of the infection is not possible and the severity of existing damage cannot be reduced. They may also affect the development of immunity.

Spread of infection needs to be restricted in an outbreak within a flock, for example by isolating animals for a defined time period until their uterine discharges clear up. Appropriate measures for disposal of infected tissues are also important in order to prevent further infection of the flock. Two schemes exist in Britain, the Sheep and Goat Health Scheme in England and Wales, and the Premium Sheep Health Scheme in Scotland to ensure that farmers obtain *Chlamydia*-free sheep when purchasing replacement ewes for introduction into a clean flock. Sera from participating flocks

are screened for the presence of antibodies to *C. abortus*, and if results are negative, flocks can be accredited as EAE-free (Aitken & Longbottom, 2004).

1.13 Vaccines – old, new and developing

In the UK, the first successful EAE vaccine was developed at the Moredun Research Institute (MRI), Edinburgh in the 1950s (Littlejohn *et al.*, 1952; McEwen *et al.*, 1951). The vaccine, which was developed by McEwen *et al.* (1951), was based on formalin-inactivated whole organisms derived from infected placental material, but this proved impractical due to the large amounts of material required for this. Subsequent studies were conducted using egg grown material of a single *C. abortus* isolate (A22), yolk-sac derived. This vaccine was formaldehyde-inactivated, Falba-adjuvanted and became commercially available in 1958 by the Wellcome Foundation Ltd. It was effective in controlling EAE for approximately 20 years when abortion started reappearing in vaccinated flocks, shown in field trials by Linklater and Dyson (1979). A second *C. abortus* isolate, S26/3 (isolated from a vaccinated flock in 1979) was subsequently added to the original isolate, A22, in 1982, however this modified vaccine did not show complete protection and was eventually withdrawn in 1992.

In the early 1980s, a mutant temperature sensitive vaccine (1B isolate of *C. abortus*) was developed (Rodolakis & Souriau, 1983) that is still used in several countries today. The vaccine, 1B, was produced from a virulent AB7 isolate by nitrosoguanidine mutagenesis, producing a live attenuated strain that can grow at 35°C but not at 39.5°C, the body temperature of a sheep, and it induces a strong and specific immune response.

The live vaccine (Enzovax, Intervet UK Ltd) is one of two commercial vaccines available in the UK, the other being an inactivated whole organism vaccine (Mydiavac, Novartis Animal Health UK Ltd). Both commercial vaccines offer good protection against EAE and limit the shedding of infectious organisms. However, as with other live attenuated vaccines, there are safety issues, including possible reversion to pathogenicity as this may result in disease and abortion in the host.

There is also a risk of *C. abortus* causing serious disease and abortion in pregnant women (Buxton, 1986). Additionally, the live attenuated vaccine cannot be administered to pregnant ewes, or to ewes that are undergoing treatment with antibiotics, whereas the inactivated vaccine can be used in these situations. Although since the inactivated vaccine contains mineral oil as the adjuvant, care must be taken when handling and administering as it can cause tissue necrosis if self-injected. These disadvantages associated with the two commercial vaccines have led to a search for improved alternative vaccines based on subunit components.

Reasonably successful vaccine studies have been conducted using purified EBs (Anderson *et al.*, 1990) and chlamydial outer membrane complex (COMC) preparations in sheep (Tan *et al.*, 1990), guinea pigs (Batteiger *et al.*, 1993) and mice (Pal *et al.*, 1997). Subunit vaccine research has focused largely on MOMP, as this is the immunodominant protein expressed on the surface of EBs and RBs and it incorporates both T and B cell epitopes, which are necessary for induction of a protective immune response (Igietseme *et al.*, 2002; Pal *et al.*, 2001). MOMP has been shown to establish protection against subcutaneous challenge with infectious organisms and inactivated EBs also gave protection against *C. abortus* infection (Tan *et al.*, 1990).

Although vaccination with purified COMCs and purified MOMP preparations has been reasonably successful, there are problems associated with the bulk growth of *C. abortus* required for their preparation. The alternative of the more cost-effective recombinant protein vaccines has become a main focus of vaccine research, particularly those based on MOMP. Studies conducted by Herring and others (Herring *et al.*, 1998) using *C. abortus* recombinant MOMP, expressed as insoluble inclusion bodies in a bacterial overexpression system, showed some protection against *C. abortus* infection, although results were inconsistent. An increasing number of studies are focusing on proteins other than MOMP, facilitated by the availability of the *C. abortus* genome sequence (Thomson *et al.*, in press). Recent identification of the POMP family (Longbottom *et al.*, 1998b) of the protective COMC has provided new vaccine targets because of their presence in the outer

membrane. POMPs have been shown to be surface-exposed (Buendia *et al.*, 1997; Longbottom *et al.*, 1998a; Tanzer *et al.*, 2001), glycosylated (Vretou *et al.*, 2001) and to induce antigen-specific T cell responses (Goodall *et al.*, 2001; Halme & Surcel, 1997), all of these factors contributing to their highly immunogenic nature. Analysis of the genome sequence has shown that the six polymorphic membrane protein (*pmp*) genes originally identified belong to a much larger family of *pmp* genes that are present in all of the *Chlamydiaceae* sequenced genomes (Longbottom and Coulter, 2003). Recent sequencing of the *C. abortus* genome will aid future chlamydial vaccine research, with particular attention on the outer membrane proteins that have been identified. These proteins are translated and localised to the membrane surface and are likely to play a role in chlamydial pathogenesis (Longbottom and Livingstone, in press).

In addition to subunit and recombinant protein vaccines, some attention has focused on development of MOMP-based DNA vaccines as a way of inducing a protective response against chlamydial infections (de Sa *et al.*, 1995). DNA vaccination holds advantages over other vaccine types, as the vaccine antigen is produced *de novo* by the host cell and production is easier due to elimination of the need to cultivate infectious organisms (Krishnan, 2000). Other advantages include the improved stability of the vaccines at room temperature (RT), cheaper production costs and they are safer than live attenuated vaccines. DNA vaccination induces both cellular and humoral immune responses, although stimulation of the cell mediated response is more crucial in the resolution of chlamydial infections (Su & Caldwell, 1995). Although in general DNA vaccination has been more successful in mice than in large animals or humans (Babiuk *et al.*, 2003).

An exception is the successful protection of turkeys against *Chlamydia psittaci* infection using gene-gun based DNA immunisations (Vanrompay *et al.*, 1999a; Vanrompay *et al.*, 1999b; Vanrompay *et al.*, 2001). Delivery of plasmid DNA incorporating the MOMP gene of the avian *C. psittaci* strain into the dermis produced protection against infection and both T-helper and B cell memory responses were primed, showing that DNA vaccination is a promising strategy

capable of preventing severe clinical signs and lesions (Vanrompay *et al.*, 1999b). Recently, Hechard *et al.* (2002, 2003) have conducted a DNA vaccination experiment using a *dna-K*-encoding (heat shock protein) plasmid in pregnant and non-pregnant mice, exemplifying the value of animal models for vaccine assessment. Weak humoral responses were induced but this was insufficient to mediate specific protection in pregnant and non-pregnant mice against *C. abortus* infection (Hechard *et al.*, 2002, 2003).

Identification of new chlamydial antigens that can be used as recombinant proteins or peptides in subunit or multicomponent vaccines is necessary to produce cheaper, safer and more stable alternatives. It is equally important to consider the route and method of delivery of potential vaccines, as well as identification of suitable adjuvants, to ensure that the antigens are correctly processed and presented to the immune system to evoke the necessary protective immune response (Entrican *et al.*, 2001; Longbottom and Livingstone, in press). Chlamydial research to date suggests that an efficacious vaccine should induce a systemic $CD4^+$ Th1 response, as well as a humoral response to enhance the memory response following secondary infection.

1.14 Project proposal and aims

At MRI, *Chlamydia* research has concentrated on the ovine host, studying immune responses and pathology, and assessing vaccine efficacy. Although it is invaluable to be able to conduct studies in the natural host for *C. abortus*, disadvantages associated with this include expensive animal and husbandry costs, primarily because of the long period of gestation in sheep. As a consequence, the number of parameters that can be examined is limited in an experiment, such as routes of inoculation and adjuvants. A mouse model of chlamydial infection offers advantages over the use of the ovine host to assess vaccines and vaccine candidates. A pregnant mouse model system will reduce the time and expense associated with initial vaccine trials due to the shorter gestation period of a mouse compared to that in sheep and larger numbers of mice can be vaccinated. This allows a wider range of parameters to be examined, particularly as there are many more reagents available for mice than sheep. A non-

pregnant mouse model exists at MRI in which the protective efficacy of vaccines can be assessed by the recovery of infectious organisms from tissues. However, since *C. abortus* targets the placenta and causes abortion, a pregnant mouse model is more appropriate for assessing vaccine efficacy by a reduction in the number of abortions.

The overall aim of this project was to develop a pregnant mouse model of chlamydial abortion that can ultimately be used for assessing the effectiveness of protein and DNA-based vaccination strategies in controlling the disease caused by the zoonotic pathogen *C. abortus*. To achieve this, the key objective was to define the immune response and pathology associated with *C. abortus* infection in pregnant mice to allow comparison with infection in sheep in order to fully validate the model. Initially it was necessary to determine if abortion occurs in pregnant mice following infection with *C. abortus* (isolate S26/3), as occurs in infected sheep. Subsequent studies were then undertaken to investigate whether latent or persistent infections develop following *C. abortus* infection of non-pregnant mice, as demonstrated by a possible recrudescence of infection and abortion once mice become pregnant. If the location of the organism can be identified in mice during the latent or persistent phase, this would provide vital information for the ovine chlamydial research field. Another key objective was to determine if *C. abortus* infected mice are immune to secondary infection and abortion in the following pregnancy post-abortion, as occurs in sheep. Provision of further information regarding aspects of the protective immune response is of benefit to chlamydial research. In order to fully validate the use of a mouse model to assess vaccines against EAE, it is necessary to compare chlamydial disease in the pregnant mouse model to that in sheep and identify similarities between the two species in terms of abortion, disease progression, development of latent infections and immunity to abortion.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Source of chemicals

All chemicals and solutions were purchased from Fisher Scientific UK Ltd or Sigma-Aldrich, UK unless otherwise stated.

2.2 Preparation of inoculum

2.2.1. McCoy cell culture

Mouse fibroblast McCoy cells (Mycoplasma-free) were used for cell culture of chlamydial organisms from stocks of egg-grown *C. abortus* isolate S26/3. This was first isolated from a vaccinated ewe that aborted in Scotland in 1979 (Linklater & Dyson, 1979). McCoy cells were grown in RPMI 1640 (Gibco BRL, UK) growth medium, containing 5% (v/v) heat inactivated foetal bovine serum (FBS; Sigma Ltd., UK), 0.2 M Hepes, 0.2% (v/v) sodium bicarbonate, 25 units (U)/ml Nystatin, 5 µg/ml Gentamycin and 0.2 mg/ml Streptomycin (Gibco BRL, UK) in 225 cm^2 tissue culture flasks (Co-Star, USA). When confluent, the cells were passaged by discarding the medium and rinsing the flask twice with sterile phosphate buffered saline (PBS) that had been pre-warmed to 37°C. A trypsin/versene mixture (T/V; 1:4 T:V) was added to cover the monolayer for 10 seconds (s), before the excess was poured off, leaving a thin covering over the cells. The flask was incubated at 37°C until the cells were seen to dislodge from the flask. 7.5 ml RPMI growth medium was added to inhibit further action of trypsin on the cells. Cells were sub-cultured (ratio of 1:5) and added to new 225 cm^2 flasks with 50 ml RPMI growth medium and were incubated at 37°C in a humidified atmosphere of 5% CO_2 (37°C, 5% CO_2) for 3 days.

Cells were infected with *Chlamydia* by incubation with RPMI infection medium (2% FBS, 0.2 M Hepes, 0.2% sodium bicarbonate, 25 U/ml nystatin, 5 µg/ml gentamycin, 0.2 mg/ml streptomycin and 1 µg/ml cycloheximide) containing *C. abortus* (S26/3 isolate). Flasks were incubated at 37°C for 3-7 days. Chlamydial cultures used for the preparation of inoculum were harvested approximately 4-5 days post-infected, titrated (**Section 2.2.2**) and stored in chlamydial transport medium (CTM; 0.2 M

sucrose, 7 mM potassium dihydrogen phosphate, 5 mM L-glutamic acid, 0.0016% (w/v) phenol red) at -20°C until required.

2.2.2. Titration of *C. abortus*

For production of challenge inoculum to infect mice, titrations of the S26/3 isolate of *C. abortus* were carried out on coverslip cultures of McCoy cells. The procedure was followed up to trypsinisation of cells from the flask, as described in **Section 2.2.1**. Following this, 10 ml of RPMI growth medium was added to the detached cells, and 100 μl of the cell suspension was added in a 1:1 ratio with 0.08% trypan blue to determine the cell concentration, where dead cells take up the blue stain while viable cells do not. The number of viable cells was counted in an Improved Neubauer haemocytometer under a light microscope at x20 magnification. The cells were then diluted in RPMI growth medium to 2 x 10^5 cells/ml to obtain a monolayer overnight, or to 1 x 10^5 cells/ml to obtain a monolayer over 3 days. One ml of the cell suspension was pipetted into each Trac bottle (Sterilin, UK) containing a 1 x 16 mm glass coverslip. Air bubbles were removed to prevent cell growth on the underside of the coverslip and the "Tracs" were incubated for the appropriate number of days at 37°C, 5% CO_2.

Ten-fold dilutions of the inoculum (S26/3; 4^{th} passage yolk sac, 10^{th} passage tissue culture) were prepared in RPMI infection medium. Growth medium was discarded from confluent coverslips and 1 ml of each dilution of the inoculum was added to Tracs in duplicate, and centrifuged twice at 1100 xg for 15 min at RT to increase infectivity. Tracs bottles were then incubated for 3 days at 37°C, 5% CO_2. To detect chlamydial EBs, the coverslips were fixed in 100% methanol for 5 minutes (min) and were then transferred to 5% Giemsa-Gurr (BDH, UK) for 20 min to stain the cells. After rinsing the coverslips in tap water, and dehydrating and clearing through a series of graded acetone-xylene solutions (100% acetone; 2:1 acetone:xylene; 1:1 acetone:xylene; 1:2 acetone:xylene and 100% xylene), the coverslips were mounted in DPX (BDH, UK) on microscope slides. Coverslips were examined by light microscopy (x400) and the number of chlamydial inclusions present was recorded,

and the number of inclusion forming units (IFUs)/ml was calculated using the following formula:

IFUs/ml = n (mean of two Tracs) x reciprocal dilution factor

The inoculum was stored at -20°C in sterile CTM until required (as previously described by (Anderson, 1986; Anderson & Baxter, 1986; McClenaghan *et al.*, 1984).

2.3 Infection of mice

2.3.1 Mouse strains and mating procedure

Adult (56- to 60-day-old) CBA ($H\text{-}2^K$; inbred) and Porton (outbred) were obtained from Harlan Olac Ltd, Oxford, UK and MRI, Edinburgh (in-house breeding), respectively. The mouse strains used in each experiment are described in **Chapters 3-6**. Virgin females were mated with males (at a ratio of 1:1 or 1:2) of the same strain over 5 days in accordance with the murine oestrus cycle. Male mice were added to female cages, left overnight for approximately 16 hours (h), when females were checked for the presence of a vaginal plug. The presence of a vaginal plug designated day 0 of pregnancy.

2.3.2 Chlamydial infection of mice

Routinely, pregnant mice were challenged with 10^6 IFUs of *C. abortus* at mid-gestation (day 11), as described by Buendia *et al.* (Buendia *et al.*, 1998). The inoculum was thawed and sonicated (Ultrasonic processor, Jencons Limited, UK) to ensure resuspension of *Chlamydia* and then diluted to 5 x 10^6 IFUs/ml with 0.1 M PBS. Mice were inoculated by intra-peritoneal (i.p.) injection of 10^6 IFUs *C. abortus* in 0.2 ml 0.1 M PBS. Control mice in each experiment were injected (i.p.) with 0.2 ml of 0.1 M PBS. Following challenge, mice were weighed and clinically scored

every day for any clinical signs, such as stary coats, tottering gait and weight loss (**Table 2.1**).

Table 2.1 Clinical scoring system

Presumed febrile response (as determined by the appearance of the coat)	**Dehydration** (determined by weight loss)	**General demeanour**
0 = sleek, glossy coat	0 = weight maintained at pre-infection level	0 = bright/active
1 = ruffled coat	1 = 10% weight loss	1 = hunched
2 = stary, stiff coat	2 = 20% weight loss	1 = tottering gait
		1 = reluctant to move

Mice were checked each day for any of the above symptoms and scored in accordance with Home Office regulations. A cumulative score of 5 results in the mouse being killed, as does a score of 4 for more than two consecutive days. Note that the general demeanour scores are additive so that a mouse could score up to 3 in this category.

2.4 Tissue collection at necropsy

Generally, liver, spleen, uterus and/or foetoplacental units (depending on pregnancy status) were removed from each animal. Samples were collected into various fixatives for histological and immunohistochemical studies and into CTM to assess infection levels through bacterial recovery. Spleen tissues were collected for immunological assays. Specific details of the necropsies are described for each experiment in **Chapters 3-6**.

2.5 Collection of blood samples

Blood samples were taken pre-infection (day 10 of pregnancy) by tail snips. Fifty μl (less than 10% of total volume) of blood was removed from each animal. Blood samples were also taken at necropsy by cardiac puncture. Samples were microcentrifuged at 13000 xg for 10 min and the sera were collected and stored at -20°C for testing for specific antibodies and cytokines.

2.6 Processing of fixed tissues

Liver, spleen and reproductive tissues were fixed in 10% formal saline (4% formaldehdye, 145 mM NaCl) for 5-7 days for histological studies and immunolabelling of chlamydial antigen. Samples collected into zinc salts fixative (ZSF; 0.5% Zinc acetate, 0.5% $ZnCl_2$, 0.05% Calcium acetate, 0.1 M Tris, pH 7-7.4) and 4% w/v paraformaldehyde (in PBS) were fixed for 6-8 h at RT, trimmed and then fixed for a further 24 h (h) at RT. Tissues were processed by dehydrating through graded alcohols and then embedding in paraffin wax at 56°C. Sections were cut as required for histological and immunohistochemical analysis.

2.7 Culture of chlamydial organisms from infected tissues

Liver and placental/uterine tissues were stored in 4 ml CTM at -20°C. Immediately prior to culturing, samples were thawed and sonicated to disrupt tissues and release EBs. Ten-fold dilutions of sonicated tissues were prepared in RPMI infection medium and duplicate samples were cultured on McCoy cells in Trac bottles, as described in **Section 2.2.2**. Inclusions were stained with Giemsa and counted, with results expressed as numbers of IFUs/g of tissue.

2.8 Immunohistochemical analysis

2.8.1 Haematoxylin and eosin staining

For histological analysis, haematoxylin and eosin (H & E; Cellpath, UK) staining was conducted on formalin-fixed tissues. Tissue sections were examined by light microscopy to identify any pathological changes associated with infection.

2.8.2 Detection of chlamydial antigen

The presence of chlamydial antigen in tissue sections was assessed using an avidin-biotin-peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) protocol, as previously described (Buxton *et al.*, 1996).

Sections (5 μm; on Superfrost slides; Merck, Leicester, England) of 10% formalin-fixed tissues were dewaxed in xylene and dehydrated in a series of alcohols (100%, 75% and 50% ethanol). Sections were rinsed in distilled water (dH_2O) before 2 x 5 min washes in wash buffer (pH 7.5, 0.02 M sodium phosphate, 0.15 M NaCl, 0.045% Tween 80). All blocking steps and antibody dilutions were prepared in high salt buffer (HSB; 0.02 M sodium phosphate, 0.55 M NaCl). Non-specific binding was blocked with normal goat serum (Vector Laboratories) (1:4 in HSB) for 30 min at RT. Sheep sera, collected from *C. abortus*-infected animals, were used as the primary antibody and 100 µl of this was incubated overnight at 4°C (1/200 in HSB). Control sections were incubated with HSB only overnight at 4°C. Sections were washed for 2 x 5 min, followed by the addition of the secondary, goat anti-sheep biotinylated antibody (1/800 in HSB) to all sections for 30 min at RT. Sections were then immersed in 1% H_2O_2 (in methanol) for 30 min at RT to remove endogenous peroxidase activity. The ABC procedure was then followed according to manufacturer's instructions (Vector Laboratories), with avidin-biotin-peroxidase solution incubation for 30 min at RT. The reaction was developed with 0.05% diaminobenzidine tetrahydrochloride (DAB). Sections were washed with water and counterstained with haematoxylin and mounted under coverslips.

2.8.3 Detection and phenotypic characterisation of immune cells in ZSF sections

Monoclonal antibodies were used to determine the presence of immune cell subpopulations in liver, spleen and foetal-placental units. Optimal dilutions of each antibody (see **Table 2.2**) were applied to sections of tissues fixed in ZSF, which limits disruption to histological structure and epitopes (Gonzalez *et al.*, 2001).

Sections (5 μm) of fixed, paraffin embedded material were cut onto Superfrost Plus slides and dried overnight at 37°C. Sections were de-waxed in xylene and taken through graded alcohols (100% to 75%) into dH_2O for 5 min each. Slides were assembled into Sequenza racks, using PBS-0.05% Tween 80 (Polyoxyethylene-sorbitan monolaurate; PBST80) to seal the slides within the racks. Sections were washed for 2 x 5 min with PBST80, followed by blocking non-specific binding with 100 μl of normal goat serum per section (1:4 in HSB) for at least 30 min. Monoclonal antibodies (100μl diluted in HSB, see **Table 2.2**) were added to each section and incubated overnight at 4°C; 100 μl HSB only was also added to duplicates of tissue sections as negative controls. Sections were washed 2 x 5 min with PBST80, and endogeneous peroxidase activity was removed by incubation with 1% H_2O_2 in methanol for 30 min at RT. Biotinylated goat anti-rat antibody (mouse adsorbed) (Caltag laboratories) (1/4000 of 100 μl) was added as the secondary step for 30 min at RT. The ABC solution (100 μl) was added and bound antibody was visualised by DAB as described for chlamydial antigen detection (**Section 2.8.2**).

Table 2.2 Monoclonal antibody information for immunohistochemistry protocols

Antibody	Cell type	Supplier	Dilution
Rat anti-Ly-6G	Granulocytes	Caltag	1/250
Rat anti-CD45R (B220)	B cells	Caltag	1/750 liver/spleen 1/250 placenta
Rat anti-CD3	T cells ($CD4^+$ and $CD8^+$)	Caltag	Various
Rat anti-CD4	$CD4^+$ T cells	BD Pharmingen and Caltag	Various
Rat anti-CD8	$CD4^+$ T cells	BD Pharmingen and Caltag	Various
Rat anti-Ly49G2	NK cells	BD Pharmingen	1/100

2.8.4 T cell immunohistochemistry on frozen sections

Immunolabelling of T cell populations was carried out on snap-frozen tissue sections because the relevant determinants are not preserved in ZSF sections. This work was conducted as a Short-Term Scientific Mission (STSM) funded by the COST Action 855 at the University of Murcia [Histologia y Anatomia Patologica, Facultad de Veterinaria, Campus Universitario de Espinardo, Spain] under the supervision of Assistant Professors Antonio Buendia and Maria Rosa Caro. The aim of this STSM was to characterise $CD4^+$ and $CD8^+$ T cell and NK cell populations in mouse tissues using immunohistochemistry on frozen sections.

2.8.4.1 Freezing tissue samples

Freezing of tissue samples was carried out at MRI, and the frozen samples were transported to the University of Murcia on dry ice. Liver and placenta samples were removed from infected mice (**Chapter 6**), immersed in OCT compound (Cryomatrix, Thermo-Shandon), frozen in liquid nitrogen and stored at -70°C.

2.8.4.2 T cell immunohistochemistry protocol

Frozen sections of liver and placenta were cut at 5 μm using a cryostat and were stored at -70ºC until required. Sections were fixed in acetone for 30 min at RT and slides were washed for 5 min at RT with 0.05 M Tris-buffered saline (TBS). Endogenous peroxidase activity was inhibited by incubation with phenylhydrazine for 40 min at RT (0.05% in TBS) and sections were washed 3 x 5 min in TBS. Sections were pre-incubated with 1/100 normal rabbit serum plus 4 drops of D-avidin to prevent non-specific binding to tissues (Vector Laboratories) for 30 min at 30°C. Either rat anti-mouse CD4, rat anti-mouse CD8 or rat anti-mouse Ly-49G2 (**Table 2.2**) were incubated for 1 h at 30ºC (1/100 dilution in TBS plus 4 drops of D-biotin; Vector Laboratories). Negative controls were incubated with TBS plus biotin for 1 h at 30ºC. Following 2 x 5 min wash in TBS, sections were incubated with biotinylated rabbit anti-rat, mouse adsorbed antibody (Vector Laboratories) at a dilution of 1/250 in TBS, 1% BSA, for 30 min at 30°C. Sections were washed 3 x 5 min in TBS, ABC solution was added to the slides for 30 min at 30ºC. Following this, sections were washed 2 x 5 min in TBS and 1 x 5 min in TB (without NaCl). DAB plus H_2O_2 was then added and incubated for 5 min at RT in the dark and the reaction was stopped by adding tap water for 5 min. Slides were counterstained with haematoxylin and mounted with glycerol gelatin.

2.9 Antibody detection

2.9.1 Enzyme-linked immunosorbent assays (ELISAs)

Blood samples were collected pre-infection by tail snips and post-infection by cardiac puncture to identify antibody isotypes produced in response to *C. abortus* infection. Sera were collected by microcentrifugation at 13000 xg for 10 min and stored at -20°C until required. Each well of a 96-well flat-bottomed plate (Greiner Bio-one, Germany) was coated with 100 μl of a 6 μg/ml stock of urografin-purified EBs (the kind gift of Morag Livingstone, MRI) in 0.1 M carbonate coating buffer, pH 9.6, overnight at 4°C. The plate was then washed 3 x 5 min PBS-0.05% Tween

20 (PBST20) and non-specific binding was blocked by addition of 200 µl 10% non-fat dried milk (NFDM) in 0.2 M carbonate buffer, pH 9.6, for 1 h at 37°C and the plate was washed three times with PBST20. One hundred µl of serum samples (1/100 dilution) in 5% NFDM/PBST20 were added to triplicate wells, incubated at 37°C for 1 h and then washed 3 x 5 min with PBST. 100 µl goat anti-mouse IgG1, IgG2a or IgM antibody, conjugated to horse-radish peroxidase (HRP) (Serotec, UK) (all at 1/1000) in 5% Marvel/PBST20 was added to each well and the plate was incubated at 37°C for 1 h. Following 3 x 5 min washes, the bound antibody was detected by addition of 100 µl/well of o-phenylenediamine dihydrochloride (OPD; Sigma-Fast OPD) substrate for approximately 10 min, and the reaction was stopped with 50 µl/well 3 M sulphuric acid. Optical densities (ODs) were read at 492 nm in a microtitre plate reader (Labsystems iEMS Reader MF, UK).

2.9.2 Western blotting

2.9.2.1 Preparation and running of SDS-PAGE gels

Western blots were performed to determine the specificity of the antibody responses. Firstly, discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out, as described by Laemmli (Laemmli, 1970) using a 10% polyacrylamide separating gel (0.375 M tris-HCl, pH 8.8) and a 4% stacking gel (0.125 M tris-HCl, pH 6.8). Three hundred µg of EBs were suspended in Laemmli sample buffer containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, boiled for 3 min and added to a large central well. Ten µl of molecular mass standards (Mark 12, Invitrogen UK) was also included. Gels were run at a constant voltage of 200 volts (V) for approximately 50 min.

2.9.2.2 Semi-dry blotting

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a BioRad Semi-Dry Blotter (Transblot SD Semi-Dry Transfer Cell) at a constant voltage of 12 V for 20-30 min.

The transfer sandwich consisted of 2 filters (BioRad extra thick blot paper) soaked in Semi-Dry Transfer Buffer (25 mM Tris, 190 mM Glycine, 20% (v/v) methanol) on either side of the nitrocellulose membrane and gel. Bands were visualised by incubation with Ponceau S Stain for 2 min to confirm the success of protein transfer and to mark protein tracks and molecular marker positions.

2.9.2.3 Antibody binding and detection

Following protein transfer, membranes were incubated in 10% Marvel in TBS, pH 7.6 overnight at 4°C to block non-specific adsorption to the membranes. Membranes were added to a Biorad multiscreen system and 500-600 μl of serum samples (1/100 dilution) in 5% NFDM/ TBS-0.1% Tween 20 (TBST) were added to individual wells within the system for 1 h at RT. Samples were removed by vacuum suction and wells were washed with TBST for 3 x 5 min. HRP-conjugated goat anti-mouse IgG1, IgG2a or IgM (Serotec, UK) diluted to 1/1000 in 5% Marvel/TBST was incubated at RT for 1 h. Following 3 x 5 min washes with TBST, DAB substrate was added for 10-15 min or until a colour reaction appeared to detect proteins recognised by the antibody. The colour reaction was stopped by washing the membrane with tap water.

2.10 Cytokine analysis

To evaluate the presence of the inflammatory cytokines, IFN-γ and TNF-α, serum samples were collected pre- and post-infection and analysed using commercial murine ELISA kits (Biosource International, Belgium). Cell culture supernatant derived from mouse splenocytes stimulated with *C. abortus*-specific antigens (see **Section 2.14**) was also analysed by ELISA to determine the specificity of the response. Procedures were carried out according to the manufacturer's instructions. ODs were read at 492 nm in a microtitre plate reader.

2.11 *In situ* hybridisation

2.11.1 Summary of probe preparation

RNA probes for mRNA detection of IFN-γ and TNF-α were prepared as summarised in **Figure 2.1**, and described in more detail in **Sections 2.11.2** and **2.11.3**.

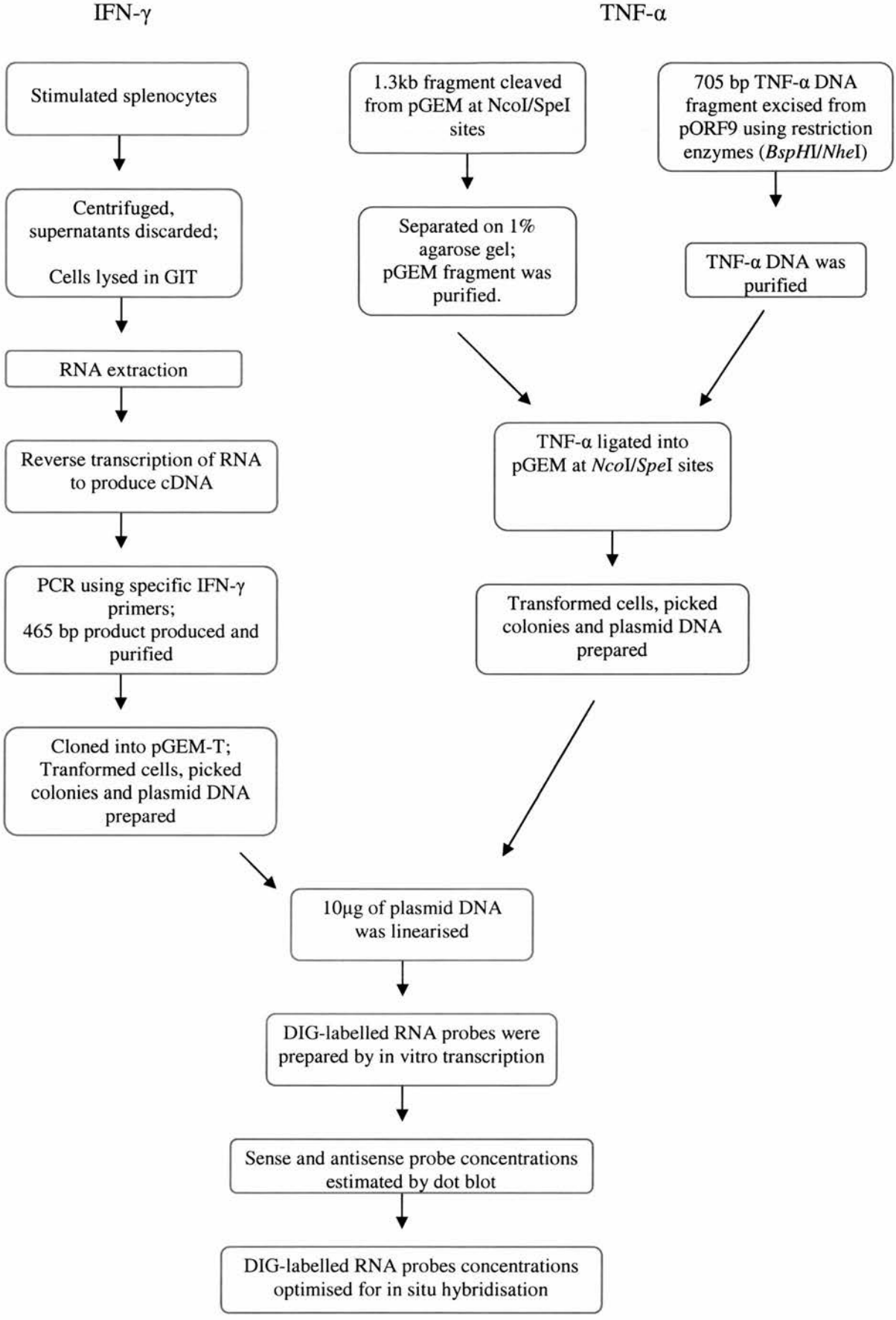


Figure 2.1 Summary flowchart of riboprobe preparation

2.11.2 IFN-γ mRNA production

2.11.2.1 IFN-γ message

IFN-γ message was obtained from mouse splenocytes stimulated with Concanavilin A (Con A). Splenocytes were prepared under aseptic conditions, as described in **Section 2.12**, and suspended at a density of 5 x 10^6 cells/ml. One ml of cells was added to each well of a 24-well plate (IWAKI, UK) containing 100 µl of Con A at 5 µg/ml, 2.5 µg/ml or 1.25 µg/ml. The plate was incubated at 37°C, in a humidified atmosphere of 5% CO_2 for 48 hours. Following incubation, the plate was centrifuged at 488 xg for 5 min, the supernatant was discarded and the cells resuspended in 600 µl guanidinium isothiocyanate to lyse the cells, and they were stored at -70°C until required.

2.11.2.2 RNA extraction and cDNA production of IFN-γ

RNA was isolated from the lysed cells using an RNeasy® Kit (Qiagen, UK), as described by the manufacturer's instructions. In brief, samples were homogenised using QIAshredder columns (Qiagen) according to the manufacturer's instructions. Ethanol was added to provide the appropriate binding conditions, and the sample was bound to a silica-gel-based membrane. Contaminants were washed away and high-quality RNA was eluted in 30 µl of water and quantified by spectrophotometry.

Reverse transcription of RNA was carried out using the AB-gene Reverse iT™ First Strand Synthesis Kit (AB-gene, UK) according to the manufacturer's instructions. Briefly, the RNA template (1 µg) and first strand primer (anchored oligo dT, 500 ng/µl) were incubated at 70°C for 5 min to denature RNA. A positive control RNA (1 µg) sample provided in the kit was included. Following this, first strand synthesis buffer, 5 mM dNTP mix plus reverse-iT™ RTase blend were added and incubated for 30 min at 47°C. Reactions were then incubated at 75°C to inactivate the RTase blend, and the concentration of the transcribed cDNA was determined by spectrophotometry.

2.11.2.3 IFN-γ DNA preparation

2.11.2.3.1 Primer and PCR design

Primers were designed for PCR amplification of IFN-γ cDNA using sequence information obtained from Medline and DNAstar PrimerSelect programmes (Accession number XM_125899). The primer sequences are shown below:

Forward: 5'-ATGAACGCTACACACTGCATCTTGG-3'
Reverse: 5'-TCAGCAGCGACTCCTTTTCCG-3'.

The PCR was optimised to produce a 468 base pair (bp) product. PCR was carried out in 100 µl reaction mixture volumes containing 300 nM concentrations of each primer, 250 µM of each dNTP, PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 2.5 U/100µl Taq DNA polymerase (Roche), with 1 ng cDNA template. Reactions were overlayed with sterile mineral oil to prevent evaporation. PCR conditions were as follows: 2 min denaturation at 94°C for 1 cycle; followed by 10 s denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 72°C for 30 cycles; and a final 7 min extension at 72°C for 1 cycle. The samples were then cooled to 4°C.

2.11.2.3.2 Agarose gel electrophoresis

PCR products were analysed by conventional agarose gel electrophoresis. One percent (w/v) agarose gels were prepared in TAE electrophoresis buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) containing 0.5 µg/ml ethidium bromide (Promega, UK). Gels were run in TAE buffer at a constant voltage of 75 V. Ten µl of PCR product plus 2 µl DNA loading buffer (30% glycerol, 0.25% Bromophenol blue, 0.05% xylene cyanol) were run alongside molecular size markers (1 kb marker, Promega). DNA fragments were visualised by UV illumination using a Hybaid transilluminator (Hybaid, UK) or an AlphaImager (GRI AlphaInnotech, UK).

2.11.2.3.3 Purification of DNA fragments from agarose gels

PCR products of 468 bp were excised from the agarose gel and purified using QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions and based on the protocol of Vogelstein and Gillespie (Vogelstein & Gillespie, 1979). Concentrations of fragments were determined by spectrophotometry and by analysis of insert alongside a DNA molecular marker at known concentrations (ΦX174/HaeIII, MBI Fermentas, UK).

2.11.2.4 Ligation of PCR products into pGEM-T cloning vector

Purified PCR products were cloned into the pGEM-T®, a T-cloning vector (Promega), which contains promoters for SP6 and T7 RNA polymerases. Use of Taq DNA polymerase (a non-proof reading enzyme) maintains a single deoxyadenosine at the 3' end of the PCR product, which allows ligation with single 3'-T overhangs of the T vector. The gel-purified PCR products were ligated into pGEM-T overnight at 4°C at various molar ratios of insert:vector [6:1, 3:1, 1:1 and 1:3]. Insert DNA was incubated with T4 DNA ligase buffer (30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), containing 50 ng of vector, insert DNA at varying ratios, and 1 Weiss U/µl T4 DNA ligase (Promega) in a total reaction volume of 10 µl. The following formula was used to estimate the amount of insert DNA required for the ligation reaction:

ng insert required = [(ng vector/ vector size (kb))] x insert size (kb) x molar ratio of insert:vector

Ligation mixes (**IFNG1**) were then transformed into competent XL-1 blue cells (**Section 2.11.2.5**)

2.11.2.5 Transformation of competent cells

PCR fragments and restriction endonuclease digested fragments were ligated into pGEM-T and transformed into Epicurian Coli® XL-1 Blue competent cells (*Escherichia coli* strain; Stratagene, UK). This was for the purpose of propagation and storage, and for the production of sufficient plasmid IFNG1 DNA to linearise for *in vitro* transcription from the T7 and SP6 promoters incorporating digoxigenin (DIG) UTP to produce RNA probes.

The transformation was carried out according to the manufacturer's instructions. Briefly, XL1-Blue cells (retrieved from -70°C) were thawed on ice and 50 µl aliquots were dispensed into pre-chilled eppendorf tubes. Twenty-five ng of ligated DNA or 0.1 ng of a positive control was added to aliquots of cells, and these were incubated on ice for 20 min. Cells were then transformed by heat shock at 42°C for exactly 45 s. Cells were then placed on ice for a further 2 min immediately after the heat shock before adding 0.9 ml SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Transformants were then allowed to recover by shaking incubation for 1 h at 37°C and 100 µl of each were plated out on L-agar plates containing ampicillin (50 µg/ml final concentration (fc)), X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; 80 µg/ml fc) and IPTG (isopropyl-1-thio-β-D-galactopyranoside; 0.5 mM fc). X-gal (lactose analogue) and IPTG (inducer of the *lac* operon) were added to allow blue-white colour screening of colonies. The multiple cloning site of pGEM-T is in the α-peptide region of the β-galactosidase gene. Insertional inactivation of the α-peptide allows recombinant clones to be selected by colour screening on X-gal/IPTG plates. Non-recombinant colonies (no insert) synthesise β-galactosidase, which breaks down X-gal to produce a blue product, i.e. resulting in blue colonies, whereas recombinant colonies (containing insert) synthesise an inactive form of the enzyme and remain white in colour. Plates were incubated overnight at 37°C, and white colonies were picked the following day. Colonies were amplified by inoculation into 10 ml Luria-Bertani (LB) broth containing ampicillin (50 µg/ml) and incubated overnight in a shaking incubator at 37°C.

2.11.2.6 IFN-γ plasmid DNA preparation

2.11.2.6.1 Determination of insert presence and size

After selecting positive colonies and culturing overnight at 37°C, 8 ml of the overnight cultures were centrifuged at 1770 xg for 10 min at 4°C to pellet cells [remaining 2 ml were used for preparing glycerol stocks; see **Section 2.11.2.6.4**]. Plasmid DNA was prepared using a QIAprep® miniprep kit (Qiagen), according to manufacturer's guidelines, to verify the presence of an appropriate insert.

QIAprep protocols are based on the modified method of Birnboim and Doly (Birnboim & Doly, 1979), where bacterial cells were lysed in 0.2 N NaOH, 1% SDS buffer in the presence of RNase A. Neutralisation of the lysate is followed by precipitation of chromosomal DNA by the addition of guanidine-hydrochloride. The precipitate is pelleted by centrifugation at 9000 xg, and the supernatant containing the plasmid DNA is adsorbed onto a silica-based gel by centrifugation in the presence of high salt. After washing, plasmid DNA is eluted in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE elution buffer).

2.11.2.6.2 Restriction enzyme digestion of DNA

Restriction enzymes (all from Roche Diagnostics Ltd, UK) were used with appropriate incubation buffers, as recommended by the manufacturer. Generally 10 U enzyme/μg DNA was used per reaction. Enzymes were incubated with DNA at the appropriate temperature for 3-18 hours, depending on the enzyme.

2.11.2.6.3 DNA production of IFNG1

Larger plasmid DNA preparations were also carried out using a QIAprep® midi-prep kit (Qiagen), in order to obtain at least 10 μg DNA for preparation of probes for *in situ* hybridisation. Fifty ml LB-ampicillin cultures were grown overnight at 37°C from stocks of 10 ml LB cultures and centrifuged at 6000 xg for 15 min at 4°C.

Plasmid DNA was eluted in 200 µl of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C until required.

2.11.2.6.4 Preparation and storage of glycerol stocks

For storage of specific colonies, glycerol stocks were prepared using 2 ml of overnight 10 ml LB cultures. Eight hundred and fifty µl of each culture was added to 150 µl of glycerol and stored at -70°C until required.

2.11.3 TNF-α mRNA production

2.11.3.1 TNF-α DNA preparation

TNF-α DNA was purchased already cloned into the pORF9 vector (Invitrogen, UK). The vector was reconstituted and streaked out according to the manufacturer's instructions. A single colony was picked and cultured in 10 ml of LB broth at 37°C overnight. QIAprep® miniprep kits (**Section 2.11.2.6.1**) were utilised to obtain plasmid DNA. TNF-α DNA was excised from pORF9 by overnight digestion with *BspHI* and *Nhe*I at 37°C, to produce a fragment of 705 bp in length.

2.11.3.2 Ligation of DNA fragments into pGEM

TNF-α was cloned into pGEM. In this instance, the cloning procedure was slightly different as the insert was not a PCR product and therefore could not be cloned into pGEM-T using the T-overhangs, summarised in **Figure 2.2**. A *Nco*I and *Spe*I fragment of pGEM was excised, leaving the pGEM with the SP6 and T7 promoter sites and this was gel-purified, as described in **Section 2.11.2.3.3**. The overhangs generated by *Nco*I and *Spe*I are compatible with those produced by the *BspHI* and *Nhe*I enzymes used to excise TNF-α from pORF9. The gel-purified TNF-α fragment was ligated with the vector fragments at the molar ratios 3:1 and 1:1 overnight at 4°C, using T4 DNA ligase enzyme (**Section 2.11.2.4**) to produce **TNFA1**.

Ligations of TNFA1 were then transformed using XL1-Blue cells, and positive colonies selected by blue/white screening, as previously described in **Section 2.11.2.5**. Four white colonies containing the insert were picked and cultured overnight in 10 ml LB broth at 37°C. TNFA1 plasmid DNA was prepared using QIAprep miniprep kits and the TNF-α fragment was excised using *Apa*I and *EcoR*I, incubated at 37°C overnight. This was analysed on an agarose gel alongside uncut plasmid DNA.

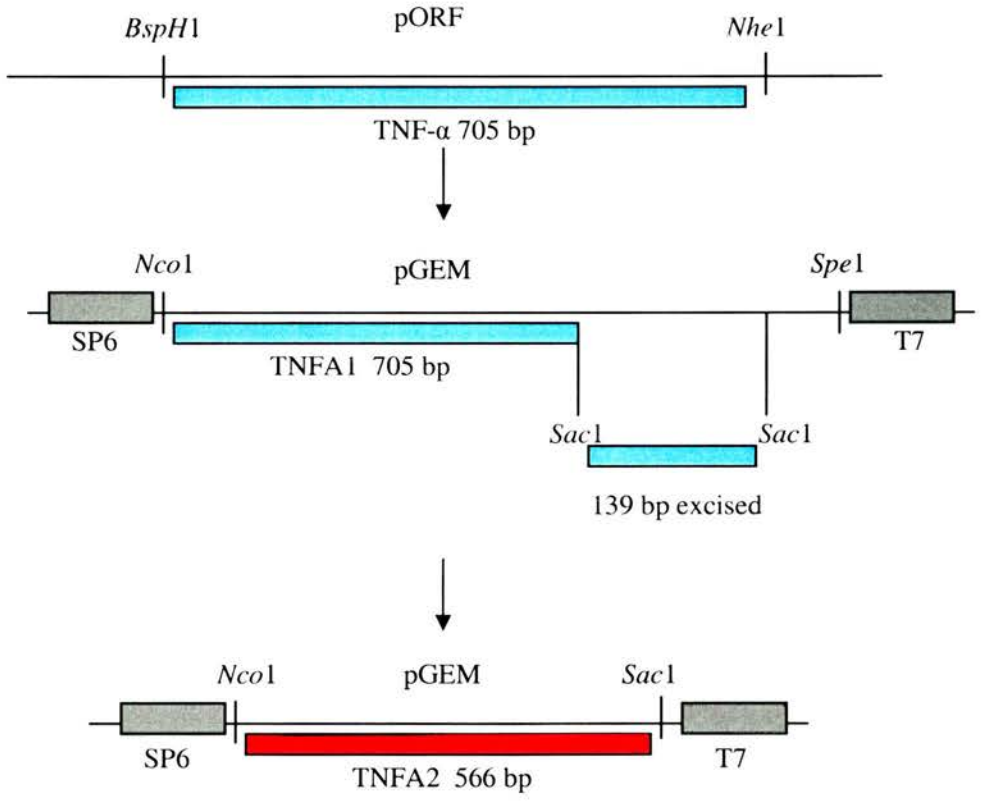


Figure 2.2 TNF-α DNA preparation from pORF to pGEM showing an outline of the steps used to produce the final TNFA2 fragment of 566 bp, used to produce mRNA probes.

2.11.3.3 Production of TNF-α-short DNA (TNFA2)

For *in situ* hybridisation studies, a maximum probe length of approximately 500-600 bp is recommended. Therefore, it was necessary to reduce the length of the 705 bp insert prior to generating RNA probes, as illustrated in **Figure 2.2**. This was achieved by digestion of the TNFA1 with *Sac*I for which a single site was present in both the TNF-α insert and the multiple cloning site of the vector, allowing removal of 139 bp at the 3' end of the insert to produce a truncated version of 566 bp, **TNFA2**. After digestion, the plasmid TNFA2 was gel-purified from the cleaved 139 bp fragment and plasmid TNFA2 was re-ligated overnight at 4°C. XL1-Blue cells were then transformed and positive colonies picked to screen for the insert, as previously described (**Section 2.11.2.5**). Ten ml LB overnight cultures, followed by QIAprep kits were utilised to obtain plasmid DNA to check for the insert by restriction enzyme digestion (*Apa*I/*Sac*I incubated overnight at 35°C). A larger preparation of plasmid DNA was carried out, using the Qiagen maxiprep kit. Plasmid DNA was eluted in 150 µl of elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C until required.

2.11.4 DIG Labelling of IFN-γ and TNF-α mRNA probes

Ten µg of plasmid DNA (IFNG1 and TNFA2) was linearised using restriction enzymes at sites upstream and downstream of the cloned insert (*Nco*I/*Sal*1 for IFN-γ, and *Apa*I/*Sac*1 for TNF-α, all incubated overnight at 37°C except *Apa*I, which was at 30°C). Both sense and antisense RNA probes were prepared by *in vitro* transcription from the T7 and SP6 promoters incorporating DIG UTP (Boehringer Mannheim, USA). One µg of linearised DNA was transcribed to produce full-length DIG-labelled RNA with T7 and SP6 polymerases. The efficiency of DIG-labelling and the concentration of the RNA probes were determined. This was conducted with a spot assay and direct detection of probes with anti-DIG, Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics Limited), and colour substrates [0.3 mg/ml nitroblue tetrazolium (NBT) and 0.16 mg/ml 5-bromo-4-chloro-3-indoyl phosphate

(BCIP)]. This was compared to DIG-labelled control RNA provided by Boehringer Mannheim.

2.11.5 *In situ* hybridisation

In situ hybridisation to detect mRNA expression of IFN-γ and TNF-α was carried out on 4% paraformaldehyde-fixed sections (Anderson *et al.*, 2001). Sections (6 μm) of fixed, paraffin embedded material were cut onto Superfrost Plus slides and dried at 37°C overnight. Sections were de-waxed in xylene twice and then taken through graded alcohols (100% to 75%), into dH_2O and then PBS for 5 min each. All subsequent washes were carried out using the Hybaid wash module (Hybaid, Ashford, England), unless otherwise stated.

Sections were washed at RT for 20 min with 0.2 M HCl and then for 1 h with 2X SSC (Saline-sodium citrate buffer; 0.15 M NaCl, 15 mM sodium citrate, pH 7.4), 5 mM EDTA at 50°C. Slides were placed into the Omnislide thermal cycler (Hybaid) and 200 μl of proteinase K (15 or 20 μg/ml; Roche Diagnostics Limited) solution in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4, was added to each section and incubated at 37°C for 15 min. This increases accessibility to the target sequence by digesting the surrounding protein. Sections were fixed in 4% paraformaldehyde at RT for 4 min, and then washed twice for 1 min at RT with PBS. All subsequent washes were carried out at RT unless otherwise stated. Slides were washed with PBS/5 mM $MgCl_2$ for 15 min and then acetylated for 10 min with 100 mM tri-ethanolamine pH 7.0, 0.25% acetic anhydride. Slides were then washed 2 x 1 min and 1 x 15 min with PBS.

Sections were prehybridised in 65 μl 6X SSC, 45% de-ionised formamide, 5X Denharts solution and 256 μg/ml bovine liver RNA for 1 h at 52°C. Sections were then rinsed in 2X SSC and incubated overnight at 52°C in hybridisation solution comprising 48% deionised formamide, 5X Denharts solution, 1000 U/ml Heparin, 0.1% Triton X-100, 24 mM EDTA, 24 mM Pipes, 720 mM NaCl, 7.2% dextran sulphate, 512 μg/ml bovine liver RNA and the DIG-labelled RNA probes (0.02-0.2

ng/ml). Easiseals (Hybaid) were used to prevent evaporation during the overnight hybridisation.

Unbound probe was removed by washing with 6X SSC containing 45% formamide for 30 min at 42°C and twice with 2X SSC for 5 min at RT. 200 µl of RNase solution (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA containing 0.1 µg/ml RNase (Dnase-free; Roche) and 100 U/ml RNase T1 (Roche)) was added and incubated for 30 min at 37°C to remove residual unbound and endogenous RNA. Sections were then washed for 2 x 5 min with 2X SSC and once with 0.2X SSC for 30 min at 50°C.

Slides were immersed in DIG buffer 1 (0.1 M Maleic acid, 0.15 M NaCl) for 1 min, followed by 200 µl blocking solution (1X blocking reagent (Roche), 0.03% Triton X-100, 0.1 M maleic acid, 0.15 M NaCl) for 30 min and then washed in DIG buffer 1 for 1 min. Two hundred µl of conjugate solution (anti-DIG antibody Fab fragments conjugated to alkaline phosphatase) were added to each section and was incubated for 2 h. Slides were washed for 2 x 15 min in DIG buffer 1 and then for at least 2 min with DIG buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Colour reactions were performed overnight at 4°C in the dark with 200 µl of DIG buffer 3 containing 0.3 mg/ml NBT, 0.16 mg/ml BCIP and 0.3 mg/ml levamisole.

Sections were washed with DIG buffer 4 (0.01 M Tris-HCl, 1 mM EDTA, pH 8.0) for 10 min and rinsed twice with dH_2O. Sections were counterstained with haematoxylin and mounted with Apathy's mountant. Sections probed with labelled sense RNA were used as negative controls to check for specificity.

2.12 Preparation of mouse splenocytes

Mouse spleens were collected into mouse wash medium (MWM; Calcium and magnesium free-HBSS (Gibco, UK) containing 2% FBS, 100 U/ml Penicillin, 0.1 mg/ml Streptomycin, 25 U/ml Nystatin, 20 µg/ml Gentamycin) under aseptic conditions and splenocytes were prepared. Spleens were disrupted and repeatedly aspirated with MWM to release splenocytes. Splenocytes were resuspended into

10ml MWM and centrifuged at 385 xg for 5 min. Supernatants were discarded, the pellet was resuspended in 1 ml of lysis buffer (0.02 M Tris-HCl, 0.75% (w/v) NH_4Cl, pH 7.2) and incubated at 4°C for 10 min to lyse the red blood cells. Following this, 9ml MWM was added to the lysed cells and centrifuged at 238 xg for 5 min. After discarding the supernatant, the lymphocytes were resuspended in 2 ml RPMI Complete medium (5% FBS, 100 U/ml penicllin, 0.1 mg/ml streptomycin, 1 mM glutamine, 100 µg/ml gentamycin, 50 µm β-mercaptoethanol, 0.08% sodium bicarbonate and 25 mM Hepes). Viable cells were counted by dye exclusion in 0.1% nigrosin using a modified Neubauer chamber. Cell concentrations used for each immunological assay are described in **Sections 2.13-2.15**.

2.13 Lymphocyte stimulation assays

Lymphocyte stimulation assays (LSAs) were used to determine the ability of defined antigens to stimulate proliferation of lymphocytes prepared from spleens of control and infected mice. Splenocytes were prepared as described in **Section 2.12** and cells were diluted to 2 x 10^6 cells/ml in complete RPMI. One hundred µl of cell suspension was added to each well of a flat-bottomed 96-well plate containing 100 µl heat-killed (HK) or triton-X-100 (TX) treated EB suspension, Con A or medium alone (see **Table 2.3** for template and concentrations). Plates were incubated for 48 h at 37°C prior to assessing cell proliferation. At 48 h, 50 µl complete medium containing 50 Bq ^{3}H-thymidine (Amersham Pharmacia Biotech) was added to each well to define activity and plates were incubated for a further 16-18 h. At this point, cells were harvested to obtain cell proliferation counts/minute (Filtermate Harvester (Matrix Filter), Packard Bioscience Company, UK).

Table 2.3 LSA template

	Mouse A			Mouse B			Mouse C			Mouse D		
A Medium												
B Con A 1.25 µg												
C HK 0.6 µg												
D HK 0.3 µg												
E TX 0.6 µg												
F TX 0.3 µg												

2.14 Cytokine production to mitogen/antigen stimulation

Cytokine responses to *C. abortus* antigen were also evaluated in splenocytes derived from control and infected mice. Mouse splenocytes were prepared as previously described in **Section 2.12**. One ml of 2 x 10^6 cells/ml in RPMI complete was added to each well of a 24-well plate containing 100 µl of RPMI medium, Con A, HK EBs or TX EBs (**Table 2.4**) and plates were incubated at 37°C, 5% CO_2 for 48 hours. At this time point, plates were centrifuged for 5 min at 488 xg to collect the supernatants, which were stored at -70°C until required for analysis by ELISA, as described in **Section 2.10**.

Table 2.4 Cytokine stimulation assay template

	Mouse A	Mouse B	Mouse C	Mouse D	Mouse E	Mouse F
A Medium						
B Con A 1.25 µg						
C TX 0.3 µg						
D HK 0.3 µg						

2.15 Flow cytometry analysis

Flow cytometry (FLC) was used to characterise lymphocyte subpopulations in control and infected spleen tissue using specific cell-surface markers. Mouse splenocytes were prepared as described in **Section 2.12**. One hundred µl of 1 x 10^7 cells/ml (1 x 10^6 cells) in RPMI complete was added to each well of a round-bottomed 96-well plate. FLC Buffer (100 µl; PBS with 5% FBS, 0.1% sodium azide) was added to the cells and the plate centrifuged at 488 xg for 3 min. The supernatant was discarded and 50 µl of the appropriate antibody or control (see **Table 2.5** for full details) was added and incubated at 4°C for 30 min. FLC buffer was added to each well (100 µl/well) and the plate was centrifuged at 54 xg for 3 min. The supernatant was then discarded and each pellet resuspended in 200 µl of FC buffer. After an additional two washes, 50 µl of PE labelled goat anti-rat IgG (1/200) was added to specific mAbs and or 50 µl of goat anti-hamster IgG (1/15), as appropriate was added

(**Table 2.5**), and incubated at 4°C for 30 min. Control wells received only secondary conjugated reagents. Cells were washed three times in 150 µl FLC buffer and fixed in 1% paraformaldehyde, after which plates were sealed and stored at 4°C until analysed (within one week) (Becton Dickinson FACS Calibur, BD, UK).

Table 2.5 Flow cytometry antibodies

Antibody	Type	Source	Cat No.	Dilution	Secondary
CD3	Primary	Rat	MCA500G	1/100	Goat anti-rat IgG (H&L) mouse adsorbed R-PE* (1/200)
CD4	Primary	Rat	MCA1767	1/100	
CD8	Primary	Rat	MCA1768	1/100	
CD19	Primary	Rat	MCA1439	1/50	
CD45	Primary	Rat	MCA1031G	1/100	
γδ TCR	Primary	Hamster	MCA1366	1/10	Goat anti-hamster IgG (H&L) R-PE* (1/15)

All from primary mAbs are from Serotec, UK

***R-PE** = R-Phycoerythrin Conjugated. Both secondary antibodies are from Caltag Laboratories

2.16 Diagnostic PCR

2.16.1 Optimisation of PCR

2.16.1.1. Primer and PCR design

PCR amplification of the *C. abortus* 16S rRNA gene was conducted using primers based on those used by Anderson and others (Anderson *et al.*, 1996), to produce a product of 640 bp. Primer sequences are as follows:

Forward: 16SF1 5'-GTTGAGGGAGAGTCTATGGGATATCA-3'
Reverse: 16SR1 5'-TACGACACGGATAGGGTTGAGACTATCCAC-3'

PCR was carried out in 50 µl reaction mixture volumes containing 300 nM of each primer, 200 µM of each dNTP, PCR buffer with 1.5 mM $MgCl_2$, 2.5 U/100µl Taq DNA polymerase and 5 µl of EBs at various concentrations. The thermocycler procedure was as follows: 15 min denaturation at 94°C for 1 cycle; followed by 10 s denaturation at 94°C, 1 min annealing at 59°C, 1 min extension at 72°C for 45 cycles; and a final extension for 7 min at 72°C. The samples were then cooled to 4°C.

A 10 µl sample of the amplified PCR product plus 2 µl DNA loading buffer was analysed by agarose gel electrophoresis (**Section 2.11.2.3.2**) to assess the size of the amplified products relative to those of the DNA ladder.

2.16.1.2 Titration of EBs to determine sensitivity

The detection limits/sensitivities of the PCR were determined in liver and spleen tissues. Uninfected mouse tissues were obtained from in-house stocks at MRI. Twenty-five mg pieces of liver tissue and 10 mg of spleen were prepared as recommended in the DNeasy Tissue Kit (Qiagen). Following processing of tissues through the kit, DNA was eluted in 200 µl TE buffer and stored at -20°C until required for PCR.

The following was carried out to determine the sensitivity of the PCR: **1.** PCRs were carried out on dilutions of EBs that were not processed through the kit; **2.** Dilutions of EBs were processed through the kit for comparison and the eluate collected for PCR; **3.** Liver and spleen were processed through the kit and the eluate was spiked with the different dilutions of EBs and PCR was carried out; and **4.** Liver and spleen were spiked with 5 μl of a dilution of EBs pre-processing in order to determine whether there were any losses during kit processing, determined by PCR. Five μl of dH_2O was added as a negative control to each set of samples. PCRs were set up on the various EB and EB-infected tissues (both liver and spleen) to determine the sensitivity of each tissue type.

2.16.2 Assessment of mouse tissues for *C. abortus* by PCR

Liver and spleen tissue from 45 representative animals from infected mouse groups (**Chapter 5**) were processed through the Dneasy Kit and PCRs were conducted on the eluted DNA, as described in **Section 2.16.1.1**. Five μl of eluted DNA from the kit was added to each PCR reaction. Negative and positive controls were included in each PCR run to ensure that there were no false positives produced.

2.17 Statistical analysis

Mean $\pm$ SEM were calculated for different groups each day p.i.. A general linear model was fitted to the data, which included group, day p.i. and pregnancy status as main effects and the group $\times$ day p.i. interaction. The data were too sparse to investigate satisfactorily any interactions involving pregnancy status in the complete data set. The parameters of the model were estimated using the general linear model (GLM) commands in Minitab 13. GLM was chosen as it allows statistical analysis of unbalanced data sets (data containing an unequal number of data points in each group on each day p.i.) GLM is an extension analysis of variance (ANOVA), to test the hypothesis that the means of several populations are equal. An overall analysis is advantageous as separating out the data, for example by day p.i., would reduce the ability to find any significant results. The model used with the complete data set was:

Response = (overall mean)+(group effect)+(day effect)+(pregnancy effect)+(group×day effect)+residual

P-values were calculated, showing whether the means for an individual factor are significantly different from one another. If the effect of an interaction (for example, group x day p.i.) is significant, then the effects of each factor are not all the same at different levels of the other factor (s). Where interactions were not significant, they were removed from the GLM and the model run again. The Tukey Method (Minitab 13) was used to compare confidence intervals to identify any differences between pairs of means following the GLM. Unless stated, GLM was used to analyse data for significant effects. One-way ANOVAs were used on some occasions to compare differences between groups at individual time points.

CHAPTER 3

PILOT STUDY – INVESTIGATION INTO THE OUTCOME OF *C. ABORTUS* INFECTION IN PREGNANT MOUSE STRAINS

3.1 Hypothesis

***C. abortus* infection in mice causes abortion and there is a progression of infection from maternal to foetal areas of placenta.**

3.2 Introduction

A combination of hormonal changes, inflammatory responses and pathological damage endanger survival of the foetus in infected mothers following colonisation of the ovine placenta with *C. abortus* (Buxton *et al.*, 2002; Entrican *et al.*, 2001). Despite extensive studies characterising the pathogenesis and control of *C. abortus* infections in ruminants, many questions remain unanswered. Mouse models of chlamydial abortion have been developed to increase understanding of disease pathogenesis and immune responses associated with infection (Buendia *et al.*, 1998;Buendia *et al.*, 1999;de Oca *et al.*, 2000).

There are no pathological signs of infection until day 90 of pregnancy in ovine infections, although organisms are thought to progress from the maternal to the foetal side of the placenta from day 60 onwards, at the time of maternal haematoma development (Longbottom & Coulter, 2003). *C. abortus* rapidly replicates in the placentomal hilus, leading to local necrosis and contiguous dissemination of infection to the cotyledonary and intercotyledonary placenta and opposing endometrium (Buxton *et al.*, 1990). It is important to fully understand the progression of infection, particularly what triggers movement of *C. abortus* across placental tissue in order to answer questions concerning specific aspects of infection such as latency.

Buendia *et al.* (1998) conducted a comprehensive study into the kinetics of infection with the *C. abortus* isolate AB7 in an outbred mouse strain (Swiss OF1). The timing of infection has been shown to be irrelevant to the outcome of infection in both mice and sheep studies, with abortion being the final result, despite the differences in placental structure. Buendia *et al.* (1998) monitored the progression of infection from

maternal to foetal areas of haemochorial placenta in mice. It varied between early and mid-challenged groups, although infiltration of *C. abortus* into the foetal trophoblast cells occurred at a similar time in both groups (Buendia *et al.*, 1998), agreeing with what is observed in infected ewes that are infected at different times during pregnancy (Buxton *et al.*, 1990).

Previously at MRI, non-pregnant mouse models have been used to assess vaccine efficacy by examining recovery of organisms from liver tissue. However, since *C. abortus* targets the placenta and results in abortion, a pregnant mouse model is of more value, as was first demonstrated by Buzoni-Gatel and Rodolakis (1983). At MRI, Anderson (1986) compared the virulence of chlamydial isolates, including the Scottish abortion isolate S26/3, in the mouse strains Porton, Swiss-White and CBA. These experiments were conducted in non-pregnant mice and therefore did not determine the suitability of these isolates for use in an abortion model. A pilot study was carried out in two mouse strains to initiate development of the pregnant mouse model that could be used for screening protective properties of protein and DNA-based vaccines using a reduction in abortion as the readout.

3.3 Aim

To investigate the outcome of infection with the S26/3 isolate of *C. abortus* in two mouse strains during pregnancy and to examine the kinetics of infection in the placenta.

3.4 Materials and methods

3.4.1 Mating of mice

Adult (56- to 60-day-old) CBA ($H\text{-}2^K$; inbred) and Porton (outbred) virgin females were mated with males of the same strain (1 male: 2 females), as described in **Section 2.3.1**.

3.4.2 Experimental procedure

Groups of mice were infected on day 11 of pregnancy via i.p. injection (**Section 2.3.2**) and euthanased on the days shown in **Table 3.1.** Mice were euthanased by injection of 1 ml of pentobarbitone (Euthatal; Rhone Merieux Limited Harlow, UK).

Table 3.1 Experimental plan of serial kill to monitor kinetics of *C. abortus* infection in pregnant CBA and Porton mice

Gp	Mouse strain	No. of mice	Inoculum	Day 3	Day 5	Day 6	Day 7	Day 8	Day 9
1	CBA	4	PBS	2	/	1	/	/	1
2	CBA	10	S26/3	2	2	1	1	3	1
3	Porton	4	PBS	2	/	/	/	2	/
4	Porton	12	S26/3	2	2	/	3	4	1

Note. Day signifies the day p.i. following infection on day 11 of pregnancy. The pregnancy rate was variable and therefore the PMs were not carried out as previously planned, explaining the random days that mice were euthanased.

At the necropsy, liver, spleen, and reproductive tract (uterus, placenta, and foetuses, where possible) were removed. Samples were collected in 10% formal saline for pathological studies, including H&E staining (**Section 2.8.1**) and immunolabelling of chlamydial antigen (**Section 2.8.2**). Samples of liver and placental were placed in CTM to assess infection levels through bacterial recovery (**Section 2.7**).

3.5 Results

3.5.1 Mating and pregnancy rate

Despite 30 females having vaginal plugs present, not all 30 mice had been successfully impregnated. The pregnancy rate varied between the inbred CBA strain (50%) and the outbred Porton strain (87.5%).

3.5.2 Viability of infection (abortion)

Abortions occurred in all *C. abortus*-infected mice of both strains in which pregnancy was allowed to proceed. Abortions in infected mice occurred between 6 and 8 days p.i. (days 17-19 of pregnancy), 2-3 days before normal term, as observed in uninfected control mice.

3.5.3 Viability of infection (culture of live organisms)

3.5.3.1 Recovery of *C. abortus* from liver tissue

Duplicate Trac cultures were set up to quantify the number of organisms in liver tissues from all animals, as described in **Section 2.7**. The number of chlamydial organisms recovered from mice at different time points throughout pregnancy was compared between the two strains (**Figure 3.1**).

Microbiological analysis revealed slight differences between the mouse strains in the number of inclusions recovered from the livers during infection of pregnant mice (**Figure 3.1**). The number of chlamydial organisms was highest on day 7 p.i. (day 18 of pregnancy), decreasing in both strains after this time. It appeared that there were more chlamydial inclusions in the cell monolayers infected with material from livers of the inbred strain than the outbred strain. Although there was no significant difference in the number of inclusions isolated from the liver between the two strains ($F_{1,13}$=1.66, p=0.230), nor were there any significant differences in the number of *C.*

abortus inclusions at the different time points of infection/pregnancy in either strain ($F_{3,13}$=0.99, p=0.440).

There were noticeable differences in the titres of *C. abortus* recovered from liver of pregnant and non-pregnant mice, with inclusions still present in livers of pregnant mice as late as day 8 p.i., whereas no inclusions were recovered after day 5 p.i. from livers of non-pregnant mice (data not shown). However, only one non-pregnant mouse was sampled on day 5 p.i., with no organisms detected in the liver. Non-pregnant mice, three of which were sampled on day 8 p.i., and two on day 9 p.i., were all negative for *C. abortus*. All uninfected CBA and Porton mice were negative for chlamydial inclusions.

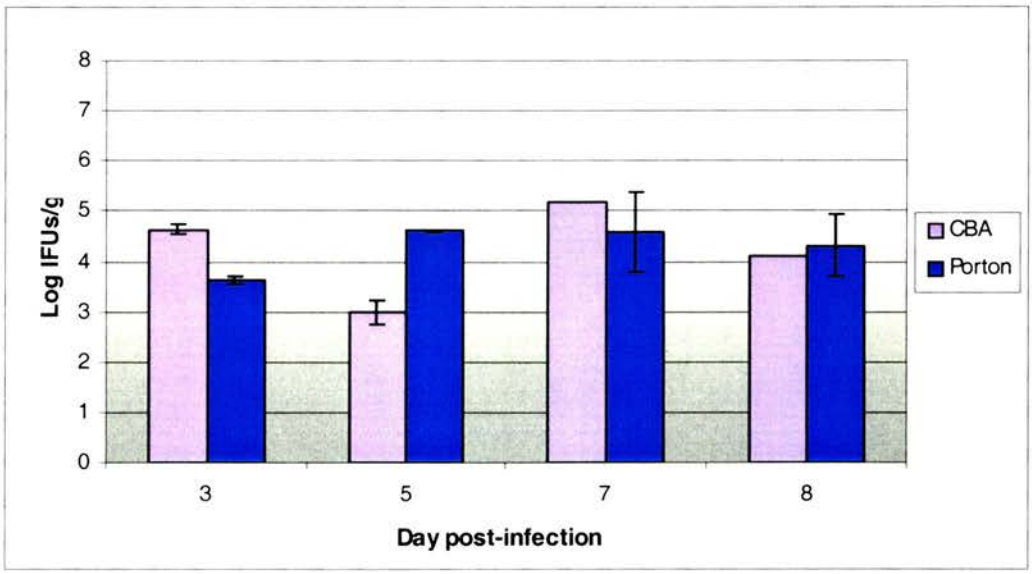


Figure 3.1 *C. abortus* isolated from livers of infected pregnant CBA and Porton mice. Numbers of inclusions (mean + SEM) were counted on McCoy cell monolayers by light microscopy, expressed as $\log_{10}$ IFUs/g of liver.

3.5.3.2 Recovery of *C. abortus* from placental tissue

Numbers of chlamydial organisms present in placental tissue at various time points during pregnancy were determined by culturing organisms from placental samples from infected mice on McCoy cell monolayers (**Section 2.7**). There were no observable trends in the number of inclusions cultured from placental tissue from infected animals of both strains over time (**Figure 3.2**). Titres of *C. abortus* were high, which is expected since the placenta is the main target organ. No statistical differences were determined in the number of IFUs/g of placenta between strains during infection/pregnancy ($F_{1,10}$=2.55, p=0.154). Data from one placental sample on 8 day p.i. suggests that the number of inclusions decreased from day 7 p.i. onwards, as also occurred in liver tissue (**Figure 3.1**). Comparison of the number of inclusions detected in placental tissue from CBA and Porton mice during infection also revealed no significant differences ($F_{3,10}$=0.46, p=0.647) in either strain. No organisms were cultured from placenta tissue of uninfected mice of both strains.

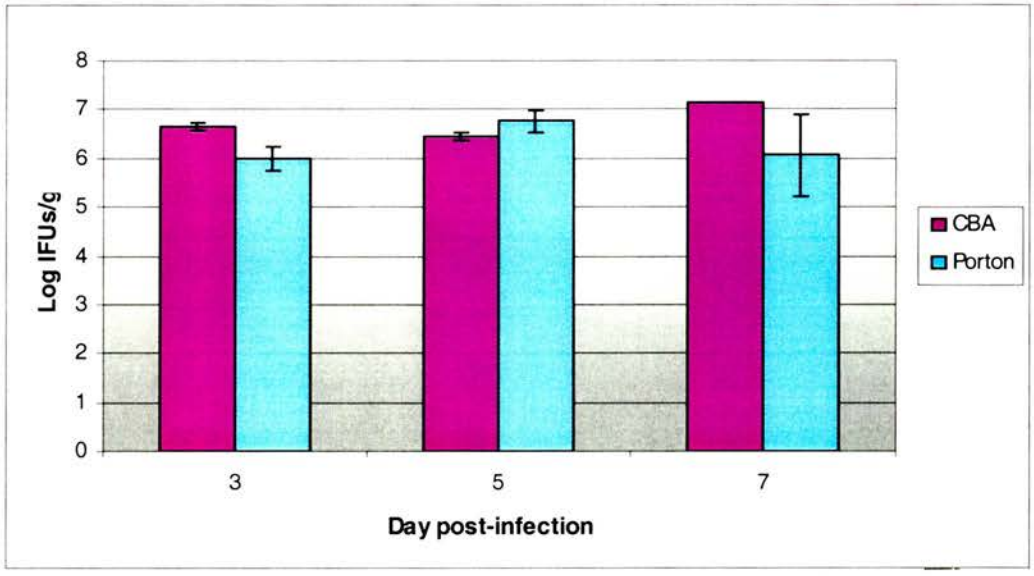


Figure 3.2 *C. abortus* isolated from placental tissue of infected CBA and Porton mice. Means (± SEM) $\log_{10}$ IFUs/g of placenta were determined by counting the number of inclusions on McCoy cell monolayers by light microscopy. Only 1 out of 4 placentas from Porton mice was obtained on day 8 p.i., with 6.6 x 10^4 IFUs/g detected. No placental tissue was recovered from the remaining 3 mice killed on day 8 p.i.

3.5.3.3 Recovery of *C. abortus*: liver versus placental tissue

Differences between *C. abortus* titres cultured from liver and placenta from both CBA and Porton mice were evaluated, as illustrated in **Figures 3.3** and **3.4**, respectively. Consistently higher numbers of organisms were isolated from placenta than from liver in the inbred strain throughout the course of infection/pregnancy. **Figure 3.3** clearly shows the significantly higher ($F_{1,8}$=76.22, p<0.001) number of IFUs/g of placental tissue compared to the number of inclusions/g of liver tissue. Similar significant differences were observed in the outbred Porton strain (**Figure 3.4**) ($F_{1,14}$=17.07, p=0.002), although these were less pronounced on days 7 and 8 p.i.

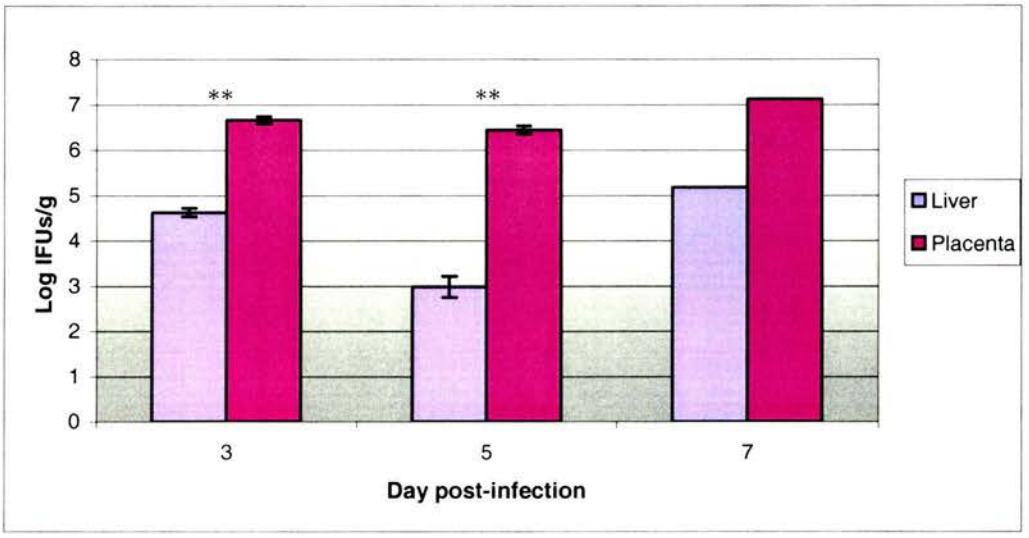


Figure 3.3 Comparison of the titres of *C. abortus* organisms in liver and placental tissue of infected CBA mice. Numbers of inclusions were estimated by counting stained cells by light microscopy and are expressed as means ± SEM log_{10} IFUs/gram of tissue. ** Significant difference p<0.01 (ANOVA). No placental tissue was recovered from the 2 pregnant mice mice killed on day 8 p.i.

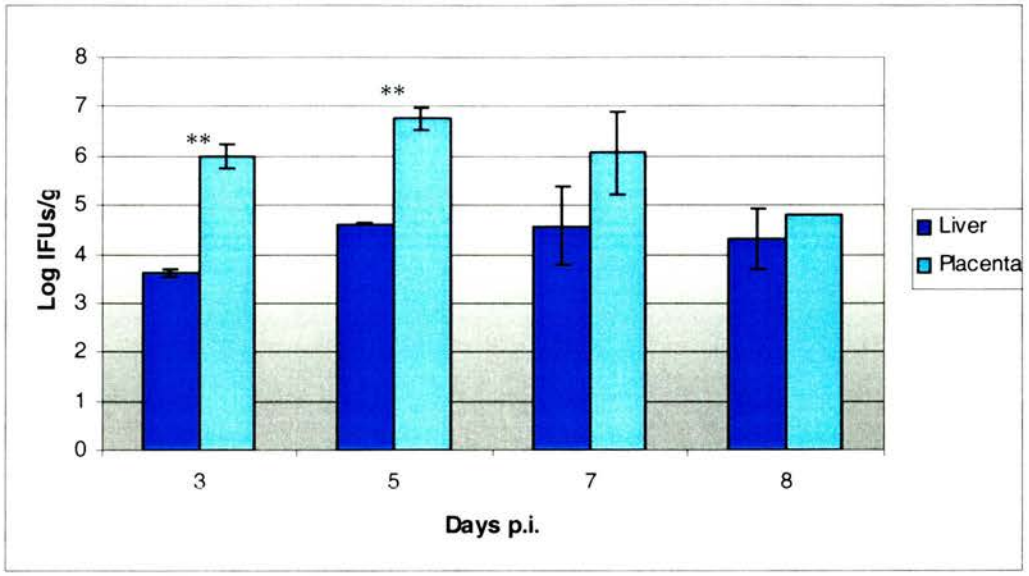


Figure 3.4 Comparison of the titres of *C. abortus* in liver and placental tissue of infected Porton mice. Numbers of inclusions (means ± SEM) were estimated by counting stained cells under light microscopy, expressed as $\log_{10}$ IFUs/gram of tissue. ** Significant difference $p<0.01$. Note: Only 1 out of 4 placentas from Porton mice was obtained on day 8 p.i.

3.5.3.4 Additional microbiological analysis

Samples of uterus were collected to identify the presence of *C. abortus* organisms from mice that had aborted. Although the sample size was small, chlamydial inclusions were recovered from mice of both strains and ranged from 4.3×10^3 to 2.6×10^5 IFUs/g of uterus, on days 7 and 8 p.i., respectively. No differences were observed between the mouse strains. The majority of animals born prematurely were cannibalised by their mothers so were not available for examination. Only one foetus was assessed for chlamydial infection by culture, and this produced negative results.

3.5.4 Disease pathogenesis in *C. abortus*-infected mice

3.5.4.1 Histological analysis

Haematoxylin & eosin staining was carried out on liver, spleen, foetoplacental units and uterine sections, as described in **Section 2.8.1**, to determine if any pathological changes had occurred. **Figure 3.5** shows examples of representative liver and placenta sections. Typically, *C. abortus* infection in mice caused an influx of inflammatory cells into tissues, as shown in **Figure 3.5a**. Inflammatory foci were scattered throughout the majority of liver sections. Although not observed in liver tissue, chlamydial inclusions were clearly noticeable in placental sections, particularly in the foetal labyrinth area (**Figure 3.5b,c**) and inflammatory cells frequently surrounded placental inclusions. No major differences were observed in histological and pathological analysis between CBA and Porton mice.

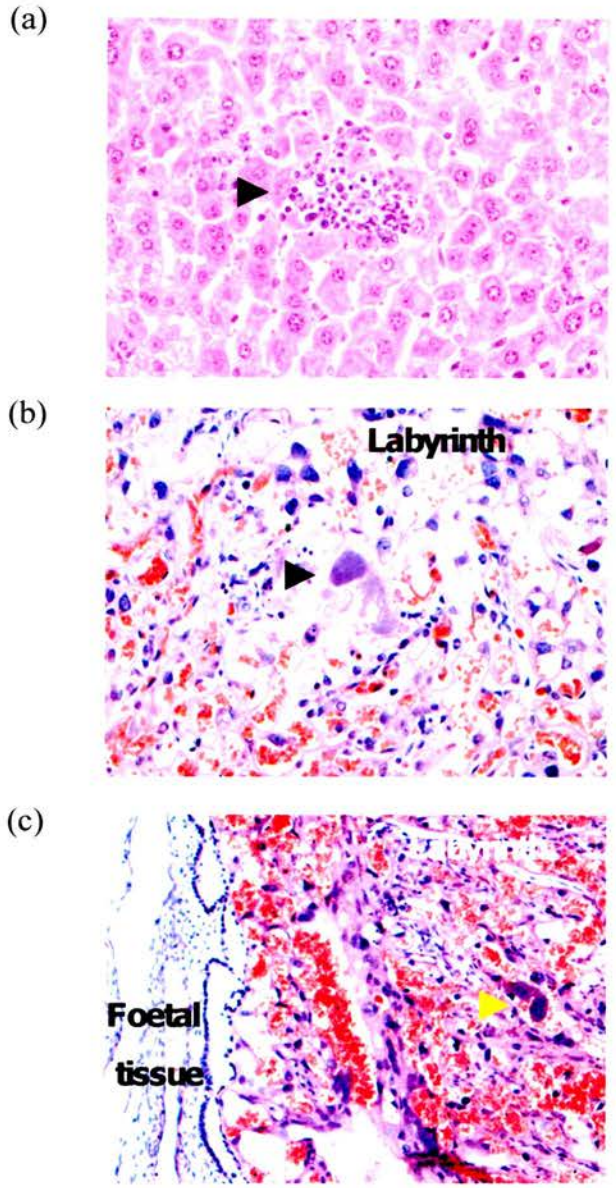


Figure 3.5 Tissue sections (H & E) from *C. abortus* infected mice. (a) Section of liver tissue from an infected CBA mouse sampled on day 5 p.i. showing an area of inflammation (arrowhead) among the hepatocytes (x200). **(b)** Section of placental tissue collected from an infected Porton mouse on day 5 p.i. with a chlamydial inclusion (arrowhead) present within the labyrinth area (x200) **(c)** Foetal side of an aborted placenta from a Porton mouse on day 7 p.i. showing inclusions (arrowhead) in the labyrinth area of the placenta (x100).

3.5.4.2 Immunohistochemical detection of chlamydial antigen

Tissue samples were labelled with polyclonal sera from *C. abortus*-infected sheep to determine whether mouse tissues had been colonised with *C. abortus* and to study the progression of the infection within these tissues. **Figures 3.6a** and **3.6b** show sections of uninfected and infected liver tissue, respectively, probed with the polyclonal sera at a 1/200 dilution.

Differences were observed between control and infected liver sections. Hepatocytes appeared to lose their organised structure within infected liver sections and had numerous inflammatory foci, the majority of which were in the same area that chlamydial antigen was detected. Chlamydial antigen was clearly visible in both liver (**Figure 3.6b**) and placental tissues (see **Figure 3.7**), indicating that there was a systemic infection and that the target organ was infected. Very few areas of chlamydial antigen were detected in liver tissue beyond day 5 p.i.. However, in all liver sections there were many inflammatory cells present throughout the infection period. It was not possible to examine spleen sections in this experiment because of the splenomegaly associated with the barbiturate used for euthanasing the mice.

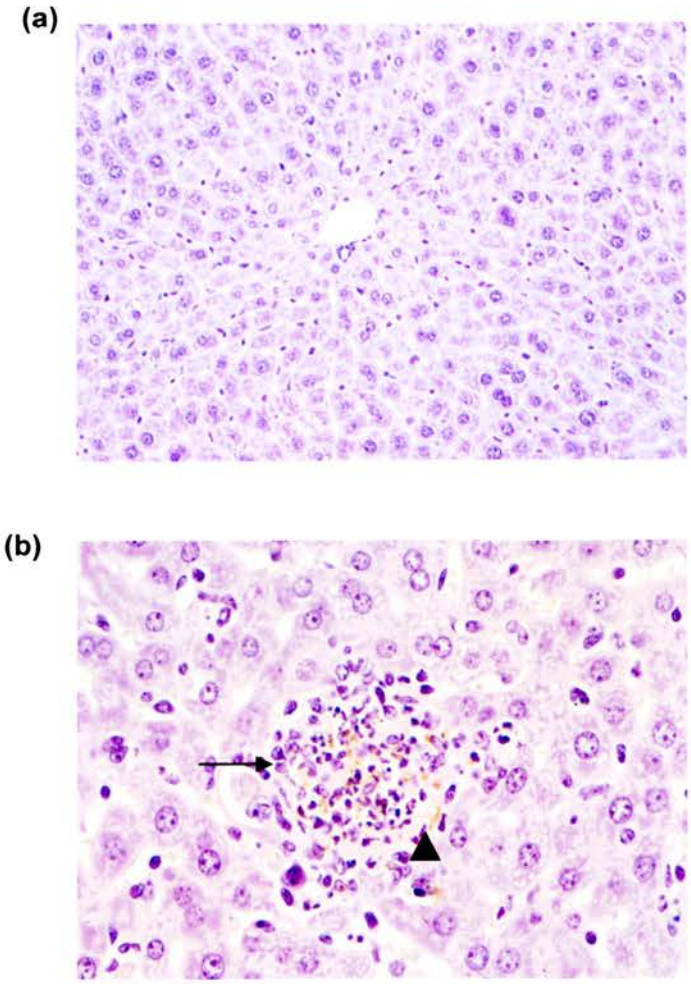


Figure 3.6 Liver sections from control and *C. abortus* infected mice following immunohistochemical detection of chlamydial antigen. (a) A control liver section from a Porton mouse killed on day 8 p.i. No chlamydial antigen was detected following incubation with the polyclonal sera (x200). **(b)** An infected liver section from a CBA mouse killed on day 5 p.i. Chlamydial antigen (brown staining; arrowhead) was detected with polyclonal sheep sera within a focus of inflammation (arrow) (x400).

3.5.4.3 Progression of infection within the placenta

There were slight differences in the localisation of infection within the placenta throughout infection/pregnancy. **Figure 3.7a** shows a placental section from a uninfected animal labelled with polyclonal sera to show the lack of infection in a negative control. This agreed with the previous culture experiments, the negative control animals having no *C. abortus* antigen present and lacking any pathological changes.

C. abortus was detected in the placental labyrinth from day 3 p.i. (day 14 of pregnancy) onwards. Chlamydial antigen was detected at the maternal-foetal interface on day 3 p.i., specifically in the giant cells and spongiotrophoblast cells. Chlamydial inclusions were scattered throughout the trophoblastic labyrinth on day 3 p.i., as demonstrated in **Figure 3.7b**. Chlamydial antigen was identified at the maternal-foetal interface in placenta samples from infected mice on day 5 p.i. (day 16 of pregnancy), similar to day 3 p.i.. As pregnancy continued and the infection progressed, inclusions were present within the foetal trophoblast cells of placental sections, close to the chorionic plate, illustrated in **Figure 3.7c**. More chlamydial antigen was stained in the foetal labyrinth than at the maternal-foetal interface at this time point. By day 7 p.i., the highest number of inclusions were observed in the labyrinth section of the placenta, with some inclusions being larger in size than previously observed. **Figure 3.7d** shows a small, heavily infected area on the foetal side of an aborted placenta, close to the foetus. Little chlamydial antigen was detected in the maternal placenta at this stage.

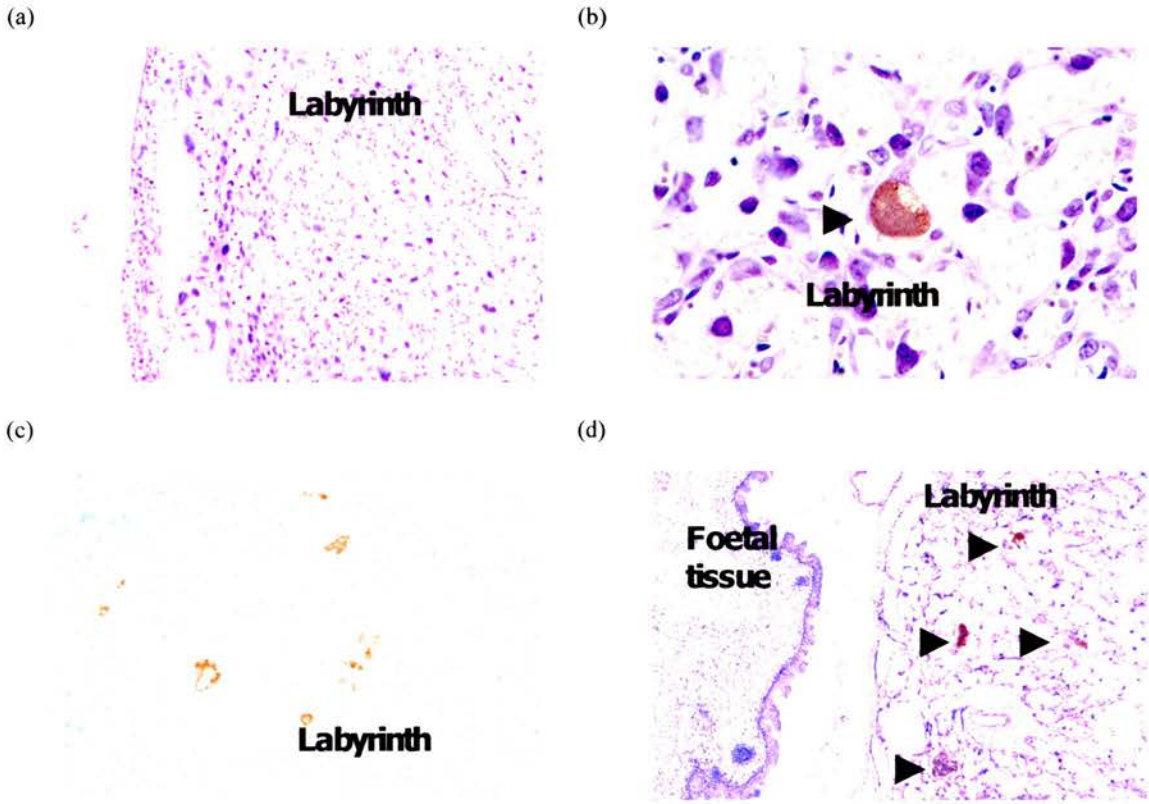


Figure 3.7 Placenta sections from control and *C. abortus* infected mice at different time points during infection/pregnancy (a) From a control mouse killed on day 8 p.i. showing the foetal side of the placenta, with no necrosis or inflammatory cells present. No chlamydial antigen was detected after immunohistochemistry (x100). **(b)** From an infected mouse killed on day 3 p.i. showing a large inclusion (arrowhead) full of EBs and RBs in the trophoblast cells of the foetal placenta (FT), detected using polyclonal sera (x400). **(c)** From an infected mouse killed on day 5 p.i. with numerous positive immunoreactions for chlamydial antigen (brown staining) detected throughout the foetal trophoblast cells and at the foetal side of the placenta (x100). **(d)** Aborted placenta from an infected mouse on day 7 p.i. with chlamydial antigen (arrowheads) scattered along the foetal side of the placenta and close to the chorionic plate at the outer edge of the trophoblast cells (x100).

3.5.4.4 Summary of kinetics of *C. abortus* infection in pregnant mice

In summary, chlamydial inclusions were observed scattered throughout the trophoblastic labyrinth between days 3 and 7 p.i. (14 and 18 of pregnancy). Inclusions were small in size and the placental sections were generally not heavily infected on day 3 p.i.. Inclusions were nevertheless present in the maternal decidua and there was slight tissue damage at the spongiotrophoblast/giant cell area. As the pregnancy continued, chlamydial antigen was observed in the maternal decidua and in the giant cells at the maternal-foetal interface on day 5 p.i.. Many inclusions were visible in the labyrinth, closer to the chorionic plate and the foetal side of the placenta on day 5 p.i., more than detected on day 3 p.i.. Immediately preceding abortion, chlamydial antigen was detected throughout the labyrinth, and scattered through the giant cell and decidual area. A summary of the kinetics of the infection in liver and placental samples is shown in **Table 3.5**.

Table 3.2 Summary of the progression of *C. abortus* infection in liver and placental tissues of pregnant mice infected at mid-gestation (day 11)

Day p.i.	Non-pregnant LIVER	Pregnant LIVER	PLACENTA Maternal Decidua	PLACENTA Spongio-trophoblasts	PLACENTA Giant cells	PLACENTA Foetal labyrinth
3	+	+	+	+	+	-
5	+	+	+	+	+	+
7	-	+	-	-	+	+
8	-	+	n/a	n/a	n/a	n/a

+ and – denote the presence or absence of chlamydial antigen by immunohistochemistry.
n/a = not applicable as all infected mice had been killed or had aborted by this time except one mouse, in which chlamydial antigen detection was very low.

3.6 Discussion

To establish the pregnant mouse model, this pilot study compared *C. abortus* infection of two mouse strains, examining the histology and immunopathology associated with chlamydial infection in pregnant animals and the progression of infection in the placenta.

An inbred strain (CBA; H-2^K) and an outbred strain (Porton) were successfully infected with *C. abortus,* demonstrated by the culture of live *C. abortus* organisms from tissues, by immunoperoxidase staining for chlamydial antigen in tissue sections, and by abortion. An inbred mouse model would allow a more direct comparison and assessment of vaccine candidates within an established system. An outbred model, however, would give a clearer indication of the range of effects of infection and efficacy of vaccines, as might occur in the natural outbred host of *C. abortus* (Aitken *et al.*, 1990). CBA (inbred) and Porton (outbred) strains were chosen because of previous studies demonstrating their susceptibility to chlamydial infection (Anderson, 1986), which revealed that Porton mice were the most susceptible to infection with abortion isolates than Swiss White and CBA mice. The susceptibility of CBA mice to abortion isolates in comparison to another inbred mouse strain, C57/BL, has also been demonstrated (Del Rio *et al.*, 2000). In contrast, this current study revealed no differences in susceptibility between the two mouse strains.

There were no significant differences between the two strains in terms of titres of *C. abortus*, distribution of inflammation or in the detection of *C. abortus* antigen. The main difference observed between the two strains was the pregnancy rate, which was 37.5% higher in Porton mice than in CBA mice, probably due to their differing breeding backgrounds. Infection of pregnant mice of both strains resulted in abortion between days 6 and 8 p.i., 2-3 days earlier than normal term of 21 days, following challenge at mid-gestation (day 11). This is similar to the pattern observed in pregnant ewes infected with *C. abortus,* which results in abortion 3 weeks earlier than normal term of approximately 145 days. The results of this current mouse experiment are comparable with those of Buendia *et al.* (1998), who infected

pregnant mice with the AB7 isolate of *C. abortus* and induced abortions on days 8 to 9 p.i.

C. abortus organisms were cultured from placental and liver tissue collected from infected CBA and Porton mice. Tissues were sampled at various time points throughout infection/pregnancy and there were no significant differences between liver titres or between placental titres of *C. abortus* during the course of infection/pregnancy in both inbred and outbred mice. Higher numbers of *C. abortus* organisms were recovered from liver and placental tissue from CBA mice on day 7 p.i., which was immediately prior to or at the time of abortion. No chlamydial organisms could be cultured from liver tissue of non-pregnant mice of either strain following day 5 p.i. and this is in agreement with work of McCafferty *et al.* (1994), who reported a decrease in the number of organisms between days 3 and 5 p.i. in *C. abortus*-infected mice. Titres decreased in pregnant animals 3 days later than in non-pregnant animals in this experiment, implying that infection is not cleared as efficiently from the liver of pregnant animals, perhaps due to immunosuppression.

Numbers of chlamydial organisms in infected placentas were high, as might be predicted for the main target organ. This implies that the organism affects the placenta in mice as it does in sheep, resulting in abortion of foetuses (Aitken *et al.*, 1990). Significantly higher numbers of organisms were observed in placental compared to liver tissue in both mouse strains. Within a placenta, infection was generally contained to one small area. In contrast, the organism was found disseminated throughout the larger liver. The same area of liver was not removed from every animal, thus in subsequent experiments, the same lobe was removed from each animal to improve comparability between animals. Samples of uterus removed from aborted animals showed numbers of organisms comparable to those recovered from liver tissue. This probably reflects the infection passing through the uterus en route to the placenta, where the highest levels of infection were detected.

In contrast to the results of McCafferty *et al.* (1994) no necrotic foci were visible by gross examination in liver or placental tissue. However, in line with their

observations, histological analysis revealed a massive influx of inflammatory cells into the liver. More inflammatory foci were observed at day 3 p.i. than at the other time points of infection/pregnancy, with an even distribution throughout the tissue sections. Inflammatory foci had decreased slightly by day 5 p.i., as observed by McCafferty *et al.* (1994), and on days 7 and 8 p.i foci had declined further.

Within placental sections, infected cells were located at the maternal-foetal interface, specifically in the giant cells, and in the foetal labyrinth area with less colonisation of the maternal placenta as infection progressed. *Chlamydia* was detected in both maternal and foetal areas of the placenta on day 5 p.i. and *C. abortus* had colonised the foetal side of placenta and the chorionic plate towards day 5/7 p.i., implying that perhaps the organism was progressing towards the foetus, but abortion occurred before there was time to infect the foetus. Initial studies by Buendia *et al.*, (1998) investigated the anatomical progression of *C. abortus* (isolate AB7) within the placenta in outbred (Swiss OF1) mice. They observed that decidual cells at the boundary of the maternal-foetal interface were the first cells to be infected and found no inclusions in the labyrinth until day 7 p.i. onwards. In contrast, we observed inclusions in the labyrinth trophoblast cells from 3 days p.i., increasing in frequency towards 7 days p.i., around the time of abortion. Buendia and others (1998) infected outbred Swiss-White mice with the *C. abortus* isolate AB7, which may indicate less susceptibly of that mouse strain compared to CBA or Porton mice, and it may also highlight differences in virulence between the abortion isolates AB7 and S26/3. As previously mentioned, Porton mice were demonstrated to be more susceptible to infection with S26/3 than CBA and Swiss-OF1 mouse strains by Anderson (1986). CBA and Porton mice were infected with S26/3 in this study, with no differences in susceptibility observed between them.

No direct foetal damage was observed in either study, although this may have been due to abortion occurring on day 7 p.i. (average), so that there was insufficient time for the organism to reach the foetus. However, Rodolakis *et al.* (1989) have isolated chlamydiae from mouse foetal tissue, although in very low numbers. The present study did not produce equivalent results, but this could be due to the low foetal

sample number that were labelled with polyclonal sera or to the poorer sensitivity of the staining method, picking up whole inclusions more frequently than free EBs. Only one foetus was used to test for the presence of *C. abortus* organisms in this current study and this proved negative. Although chlamydiae reach the ovine foetus in the final trimester of pregnancy, the time between infection and abortion is greater in sheep compared to the mouse model. It would be interesting to determine if lesions and positive immunoreactions are found in surviving offspring, as demonstrated by Baumgärtner and Bachmann (Baumgartner & Bachmann, 1992) in mice infected with *Coxiella burnetii*, suggesting that infection of foetuses occurred immediately prior to or after birth. A plausible explanation of the negativity in foetal tissue is that GMG cells of the placenta may limit the passage of *C. abortus* across the placenta to the foetus, as has been demonstrated by Stewart (Stewart, 1994) in the prevention of transmission of viruses from mother to foetus. Chlamydial inclusions have been observed in these GMG cells, both in healthy and degenerating cells (it should be noted that GMG cells do not multiply post day 15 of gestation) (Sanchez *et al.*, 1996), therefore, the infection may be prevented from progressing further. GMG cells are located in the decidua basalis and the metrial gland, and they may remove abnormal trophoblasts that contain *C. abortus*.

The liver and spleen have previously been reported as the primary target organs in systemic chlamydial infection following i.p. injection in mice, with the placenta being colonised later (Buendia *et al.*, 1998). In this study, *C. abortus* is present in both the liver and placenta of both mouse strains by 3 days p.i., but no data were available prior to this, which was similar to work by Buendia *et al.* (1998). The main differences between the two studies was the progression of infection in the placenta, the foetal labyrinth area being colonised earlier in this study compared with that identified in work by Buendia *et al.* (1998) and as previously mentioned this may be due to the different mouse and chlamydial strains used.

Infection in this experiment was associated with a maternal inflammatory cellular infiltrate in placental and liver tissue, which is discussed further in **Chapter 4**. Buendia *et al.* (1999) suggest that these are predominantly PMNs. Neutrophil

infiltration and necrosis at the maternal-foetal interface are characteristic of *C. abortus* infection in the ruminant placenta (Buxton *et al.,* 1990) therefore it was not unexpected that this was observed in mouse infections. PMNs were involved in resolution of the primary infection, successfully eliminating *C. abortus* from the liver of non-pregnant mice by day 5 p.i. (Buendia *et al.,* 1999). Mononuclear leukocytes may also play a role, although multiplication of chlamydiae in the liver and spleen is probably controlled by neutrophils (Buendia *et al.,* 1999). However, this clearance of organisms was not observed in the liver or placental tissues of pregnant mice compared to non-pregnant mice, suggesting that the activity of these neutrophils may be less efficient in pregnant mice, perhaps being down regulated in some way. It has been reported that these polymorphonuclear cells are involved solely in the resolution of primary infection, but not in the control of secondary infection (de Oca, 2000). Neutrophils however may lead to the recruitment of other cell types, for example, $CD8^+$ T cells (de Oca *et al.*, 2000b), and influence the production of cytokines, in particular IFN-γ and TNF-α (Ellis & Beaman, 2004), as will be discussed in **Chapter 4**.

The larger-scale infection in the labyrinth, in the chorionic plate next to the yolk sac in the foetal side of the placenta, close to the time of abortion, raises the possibility that the maternal-foetal junction is disrupted in some way, inducing abortion of the foetus. Variations in hormone expression during pregnancy both in sheep and mice are highly likely to play a role in induction of abortion in conjunction with the inflammatory response. The influx of inflammatory cells to the maternal and other foetal areas of the ovine placenta may result in necrosis and therefore malfunction of the maternal placenta, leading to abortion. This is detrimental to both the mother and the foetus, but allows the ewe to develop immunity following clearance of the infection. But does this occur in mice? Questions relating to this and other factors associated with EAE were investigated using this pregnant mouse model and are discussed in the following chapters.

CHAPTER 4

IMMUNOLOGICAL RESPONSES ASSOCIATED WITH *C. ABORTUS* INFECTION IN AN INBRED MOUSE STRAIN

4.1 Hypothesis

Infection of pregnant mice with *C. abortus* results in a Th1 immune response, with $CD4^+$ and $CD8^+$ T cells recruited to sites of infection and increased IFN-γ levels in infected animals.

4.2 Introduction

PMNs and mononuclear cells, such as T cells, are the principal immune cells recruited to sites of infection in *C. abortus*-infected sheep and these contribute to the gross necrotic, inflamed and thickened nature of infected placental membranes (Buxton *et al.*, 2002). Many cells expressing mRNA encoding for TNF-α, a cytokine that is characteristic of a Th1 immune response, and none expressing IL-4, characteristic of a Th2 response, were present in infected placental tissues. Macrophages, MHC class II-expressing cells, CD4 and CD8 T cells and B cells have all been identified in the mixed inflammatory infiltrate of the intercotyledonary membranes of infected ovine placentas (Buxton *et al.*, 2002).

Depletion studies in mice have demonstrated the relative importance of some of the afore-mentioned immune cells in control of *C. abortus* infections (Buendia *et al.*, 2002; Buendia *et al.*, 2004; de Oca *et al.*, 2000). PMNs have a crucial role in the first line of defence against *C. abortus*, and are a major component of the innate immune response. Infection of PMN-depleted mice with *C. abortus* has been shown to result in an earlier mortality compared to non-depleted animals, and this was associated with higher levels of TNF-α (Buendia *et al.*, 1999). PMNs are a known source of TNF-α, and depletion of PMNs prevents the lethal inflammatory cytokine shock induced by *T. gondii* in WT mice (Marshall & Denkers, 1998). In contrast, the association of TNF-α with higher mortality in PMN-depleted mice infected with *C. abortus* shows that PMNs may not be the source of this cytokine in *C. abortus*-infected mice (Buendia *et al.*, 1999). Widespread necrosis was observed in uteroplacental units whereas extensive neutrophil infiltration was observed in infected WT mice in the absence of necrosis. Liver tissue damage was more

pronounced in infected pregnant WT mice than in non-pregnant mice (Buendia *et al.*, 1999). Recruitment of immune cells, such as $CD8^+$ T cells and macrophages, to sites of infection was decreased in the absence of PMNs, allowing further multiplication of the organism and damage to tissues (de Oca *et al.*, 2000). However, PMN depletion did not prevent predomination of a Th1 immune response characterised by IFN-γ production.

Del Rio *et al.* (Del Rio *et al.*, 2000) reported that clearance of *C. abortus* infection was associated with a prominent neutrophil response and the presence of $CD8^+$ T cells, agreeing with studies by Buzoni-Gatel *et al.* (Buzoni-Gatel *et al.*, 1992). There is controversy as to the relative importance of $CD4^+$ T cells and $CD8^+$ T cells, but it is widely documented in the literature that *Chlamydia* infections are controlled by a Th1 response, characterised by $CD4^+$ T cells, NK cells, IL-12, IFN-γ and TNF-α (Buxton *et al.*, 2002; Geng *et al.*, 2000; Morrison *et al.*, 2000). Phenotypic characterisation of immune cells in snap-frozen tissues revealed the presence of both $CD4^+$ and $CD8^+$ T cells in non-pregnant *C. abortus*-infected mice (Buendia *et al.*, 1999). $CD4^+$ T cells are considered dominant over $CD8^+$ T cells and the latter are thought not to act in the normal cytolytic manner, but to have a role similar to $CD4^+$ T cells in the recruitment of other cell types and release of IFN-γ. B cells are thought to play only a minimal role in early events of primary immune response to *C. abortus* infection, as demonstrated by B cell-deficient mice establishing a specific immune response leading to protection from secondary challenge (Buendia *et al.*, 2002). NK cells play a critical role early in the innate immune response, with depleted mice showing enhanced susceptibility to infection, higher mortality rates and increased bacterial loads in tissues (Buendia *et al.*, 2004).

Depletion of IFN-γ, the major controlling-cytokine in chlamydial infections, in mice led to increased number and size foci of inflammation within the liver (McCafferty *et al.*, 1994). Much of the IFN-γ research has focused on interactions with NK cells of the innate immune response, and macrophages and T cells, components of the secondary, acquired immune response (Ellis & Beaman, 2004). However, IFN-γ has been shown to be a potent and critical modulator of the innate immune response, and

its interaction with PMNs has been overlooked until recently. Originally thought of as 'terminal, short-lived cells', PMNs are in fact active, dynamic cells that respond to immunomodulators such as IFN-γ, which can mediate the transition between the innate and acquired immune response (Ellis & Beaman, 2004). Therefore, PMNs may play a role in the recruitment of other cell types and also facilitate killing of the invading organism.

Although numerous studies have examined immune responses to *C. abortus* infections in mice, many have utilised non-pregnant mice, and different mouse and *C. abortus* strains to those used in this model. In order to define the immune responses in this model of chlamydial abortion, pregnant mice were infected with *C. abortus* and immune responses characterised in mouse tissue samples.

4.3 Aim

To characterise the immune responses associated with *C. abortus* infection at different time points during pregnancy, and to carry out a qualitative analysis of the phenotype and distribution of immune cell subpopulations and *in situ* analysis of cytokine expression at the maternal-foetal interface and in maternal tissues.

4.4 Materials and methods

4.4.1 The Whitten effect

Due to the mating problems that occurred in the previous experiment (**Section 3.5.1**), the Whitten Effect (Gangrade & Dominic, 1984) was used to synchronise mouse oestrus cycles for timed matings. All mice used in this experiment were CBA ($H\text{-}2^K$) inbred mice. Female mice are anoestrous in the absence of males. Females were isolated for 2 weeks before being exposed to male mice for 3 days, with only olfactory and visual contact possible. Exposure of female mice to pheromones in the urine of male mice initiated the oestrus cycle and the majority of females were in oestrus on the third night after exposure. On the third night, male mice were placed

in cages with female mice (1 male: 2 females), with a total of 25 males to 50 females. Females were checked for a copulatory plug on the fourth morning, with the presence of a plug denoting day 0 of gestation (Gangrade & Dominic, 1984).

4.4.2 Experimental procedure

Sixteen female mice that mated successfully were inoculated by i.p. injection with 10^6 IFUs of *C. abortus* in 0.2 ml of 0.1 M PBS on day 11 of gestation. A further 6 non-pregnant, but mated mice, were inoculated with *C. abortus* and 16 pregnant negative control mice were injected i.p. with 0.2 ml 0.1 M PBS. Mice from each of the groups were killed on days 3, 5, 6 and 7 p.i. as outlined in **Table 4.1**.

Table 4.1 Outline of serial kill to examine immune responses in uninfected and *C. abortus*-infected pregnant mice

Group	Mouse Strain	No. of animals	Inoculum	No. of mice killed			
				Day 3 p.i	Day 5 p.i.	Day 6 p.i.	Day 7 p.i.
1	CBA, pregnant	16	PBS (control)	4	4	4	4
2	CBA, pregnant	16	*C. abortus* Strain S26/3	3	4	3	3
3	CBA, mated non-pregnant	6	*C. abortus* Strain S26/3	3	0	2	1

Note. Three extra mice in group 2 were abortion controls and left to go to term.

Mice were euthanased with CO_2, followed by cardiac puncture (to obtain blood samples (**Section 2.5**) and cervical dislocation. The uterus was rapidly removed and the pups were killed by cervical dislocation. Liver, spleen, placenta and reproductive tract samples were collected into various fixatives, as described in **Sections 2.4** and **2.8**, for pathological studies on paraffin sections, and liver and placenta samples were collected in CTM for culture of chlamydial organisms.

4.5 Results

4.5.1 Pregnancy and abortion rate

In contrast with the poor pregnancy rate (50%) observed in the CBA strain of the previous study (**Section 3.5.1**), 64% (32) of mated females became pregnant with exploitation of the Whitten effect in this study.

Three extra pregnant mice were infected with *C. abortus* on day 11 of pregnancy and these mice aborted on day 18 of pregnancy confirming the efficacy of the challenge inoculum and dose. Mice did not display any clinical signs during infection.

4.5.2 Effect of *C. abortus* infection on animal weight

Weights of infected and uninfected pregnant mice were compared between days 11 and 14 of pregnancy to determine whether infection with *C. abortus* had a detrimental effect on development of the pregnancy. No significant differences ($F_{1,24}$=0.29, p=0.597) were found between the control and experimental groups (**Figure 4.1**).

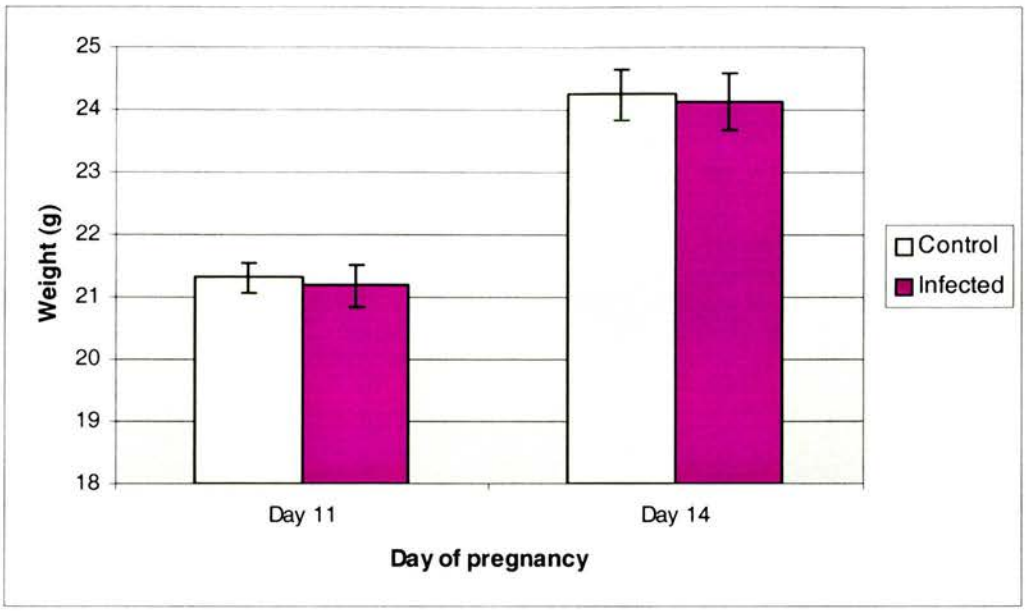


Figure 4.1 Weights of control and *C. abortus*-infected pregnant mice following inoculation on day 11 of pregnancy. Mean (± SEM) were calculated for control and infected mice on days 11 and 14 of pregnancy.

4.5.3 Culture of *C. abortus* organisms from mouse tissues

The differences in titre of organisms isolated from liver and placenta samples on McCoy cell monolayers (**Section 2.7**) were evaluated. Liver samples were titrated from 10^{-2} to 10^{-4} and placental samples from 10^{-2} to 10^{-5}, based on preliminary data discussed earlier (**Section 3.5.3**). Levels of infection were significantly higher in placenta than in liver tissue throughout infection (**Figure 4.2**) ($F_{1,24}$=65.51, $p<0.001$). Titres of *C. abortus* varied significantly during infection/pregnancy ($F_{3,24}$=4.04, p=0.036) with highest counts on day 5 p.i. in placenta and day 6 p.i. in liver. Results were comparable with those estimated in the pilot study (**Figures 3.3** and **3.4**). Only liver titres from pregnant animals are included in the figure; non-pregnant mice were still infected on day 6 p.i, but infection levels were much lower (approximately 4 fold). All tissues from uninfected mice were negative for chlamydial organisms.

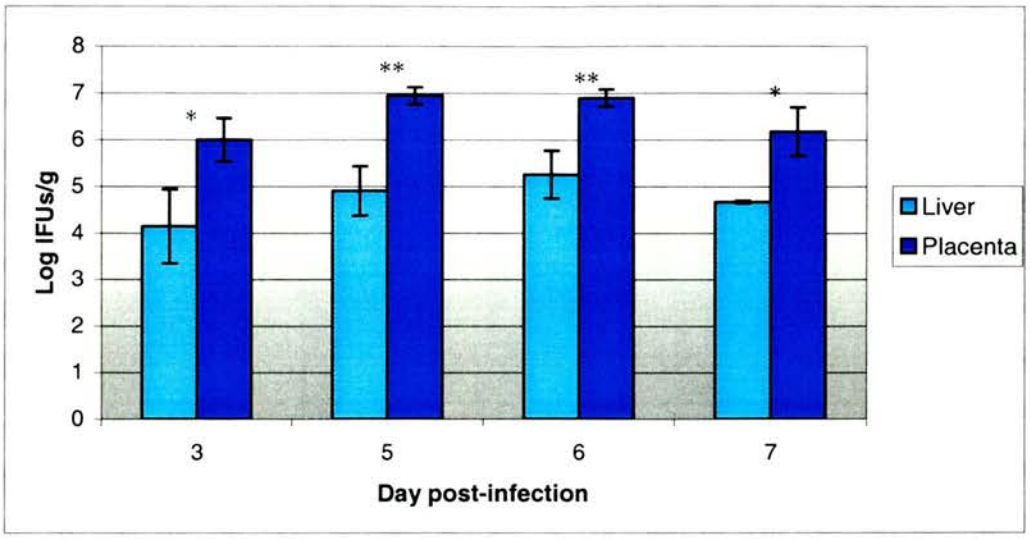


Figure 4.2 Number of *C. abortus* organisms cultured from liver and placental tissues from pregnant mice infected on day 11 of pregnancy. The number of inclusions (means ± SEM) was estimated by counting stained cells under light microscopy, expressed as $\log_{10}$ IFU/g of tissue. Significant difference between liver and placenta titres **p<0.01, *p<0.05 (ANOVA).

4.5.4 Histological analysis in pregnant and non-pregnant mouse tissues

H & E staining was carried out on liver, spleen and foetoplacental units after fixation in 10% formal saline. Examination of slides revealed an influx of inflammatory cells into areas of both tissue types, with more inflammation in liver than placenta, as observed in the pilot study (**Figures 3.6** and **3.7**). No necrosis was visible in infected liver or spleen tissues or if present, placental tissues, and tissue sections from uninfected mice had a normal histological appearance.

4.5.5 Immunohistochemistical analysis

4.5.5.1 Detection of chlamydial antigen

To examine the kinetics of infection, immunohistochemical detection of chlamydial antigen (**Section 2.8.2**) was carried out at each time point to identify any associations between the progress of infection and the immune response.

Chlamydial antigen was detected in liver tissue from day 3 to 7 p.i. in all pregnant animals that were infected (**Figure 4.3**). No significant differences in the number of areas staining for chlamydial antigen were observed over the course of infection ($F_{3,11}$=1.51, p=0.277), although there was a clear increasing trend as pregnancy progressed.

Non-pregnant animals had lower infection levels than pregnant mice (**Figure 4.3**). Levels of infection in both pregnant and non-pregnant mice were similar on day 3 p.i.. Infection subsequently increased in pregnant animals compared to non-pregnant animals throughout the infection. Small clusters of chlamydial antigen were present throughout liver tissue of infected mice, although no whole inclusions were observed. All tissues from uninfected mice were negative for chlamydial antigen.

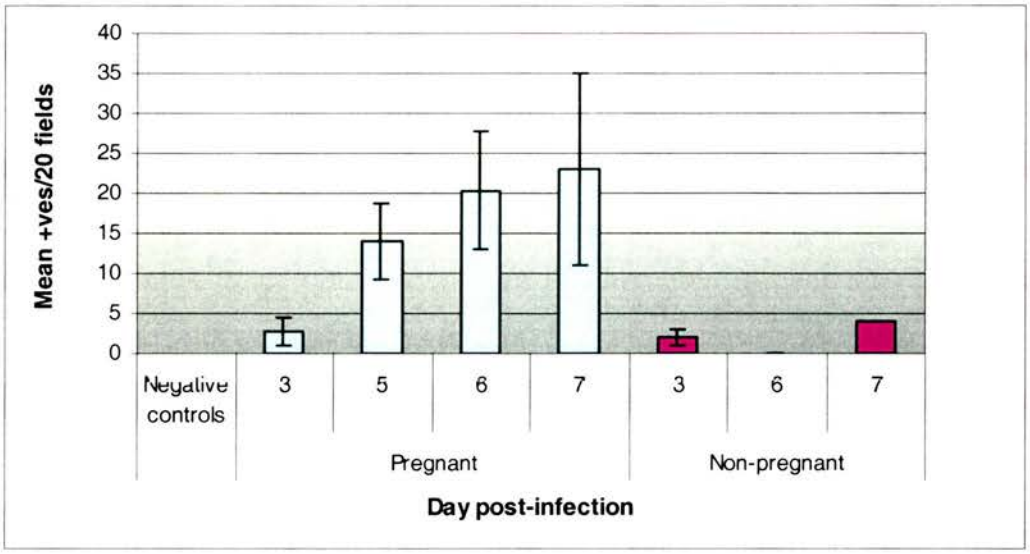


Figure 4.3 Chlamydial antigen detection in liver tissue from uninfected and *C. abortus* infected mice inoculated on day 11 of pregnancy. Numbers of positive immunoreactions (means ± SEM) of chlamydial antigen were counted in 20 fields (x20 magnification) in pregnant and non-pregnant mice. Negative control group represents pooled data from all uninfected mice.

Chlamydial antigen was detected in placental tissue of *C. abortus*-infected pregnant mice, with highest numbers observed on day 7 p.i., around the time of abortion. Chlamydial inclusions full of EBs and RBs were observed in the foetal trophoblast cells, some of which were surrounded by inflammatory cells. Other areas of infection were detected in the giant cell layer and the maternal decidua, with both extracellular EBs and whole inclusions present. *C. abortus* progressed from the maternal to the foetal side as observed previously in the pilot study (**Figure 3.7**), with no significant differences between time points ($F_{3,10}$=1.33, p=0.331) (**Figure 4.4**).

Numbers of chlamydial antigen foci counted on day 3 p.i. in infected placental tissue sections (**Figure 4.4**) were comparable to the highest number counted in infected liver tissue sections on day 7 p.i. (**Figure 4.3**). This demonstrates the higher susceptibility of placenta than liver tissue to *C. abortus* organisms and agrees with previously calculated bacterial burdens (**Figure 4.2**). Numbers of chlamydial antigen in placenta were consistently higher than those observed in liver throughout infection/pregnancy ($F_{1,23}$=20.81, p<0.001).

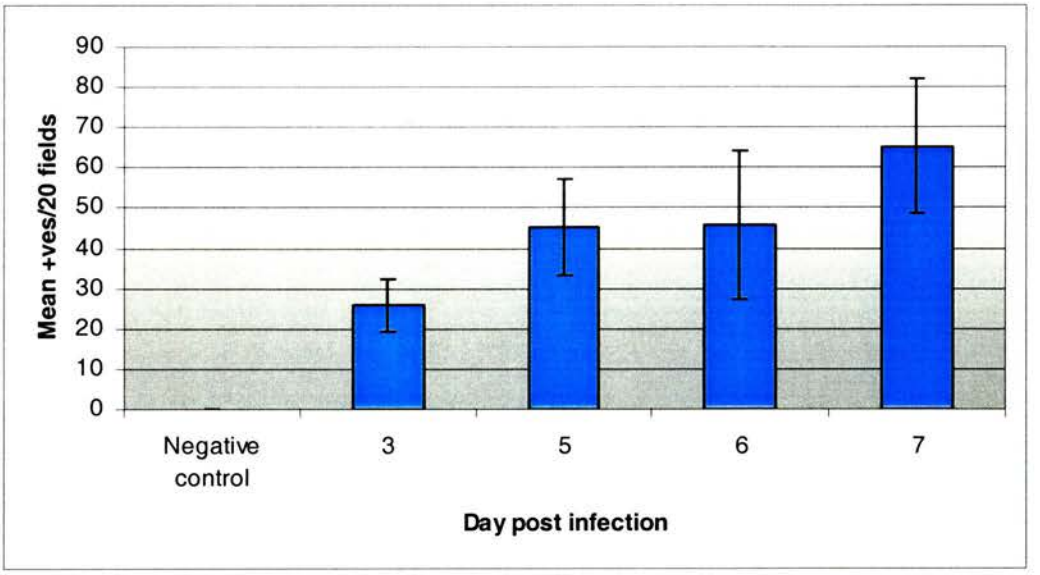


Figure 4.4 Chlamydial antigen detection in placental tissue from control and *C. abortus* infected mice. The number of positive immunoreactions (means ± SEM) for chlamydial antigen was counted in 20 fields (x20 magnification). Negative control group represents pooled data from all uninfected mice.

4.5.5.2 Detection of Ly-6G$^+$ cells in tissue sections

To examine the role of the acute inflammatory response to infection, Ly-6G, as a marker for PMNs, was immunolabelled in ZSF-paraffin wax sections, as described in **Section 2.8.3**.

Ly-6G$^+$ cells were observed in infected liver tissues both in inflammatory foci and as individual cells (illustrated in **Figure 4.5a-c**). Larger foci were present on days 3 and 5 p.i., but the size and number of foci had decreased by day 7 p.i., with individual positive cells being more evident. Occasional positive cells were detected in control liver sections (**Figure 4.5d**), but no inflammatory foci were present and liver structure was undisrupted in comparison to infected sections.

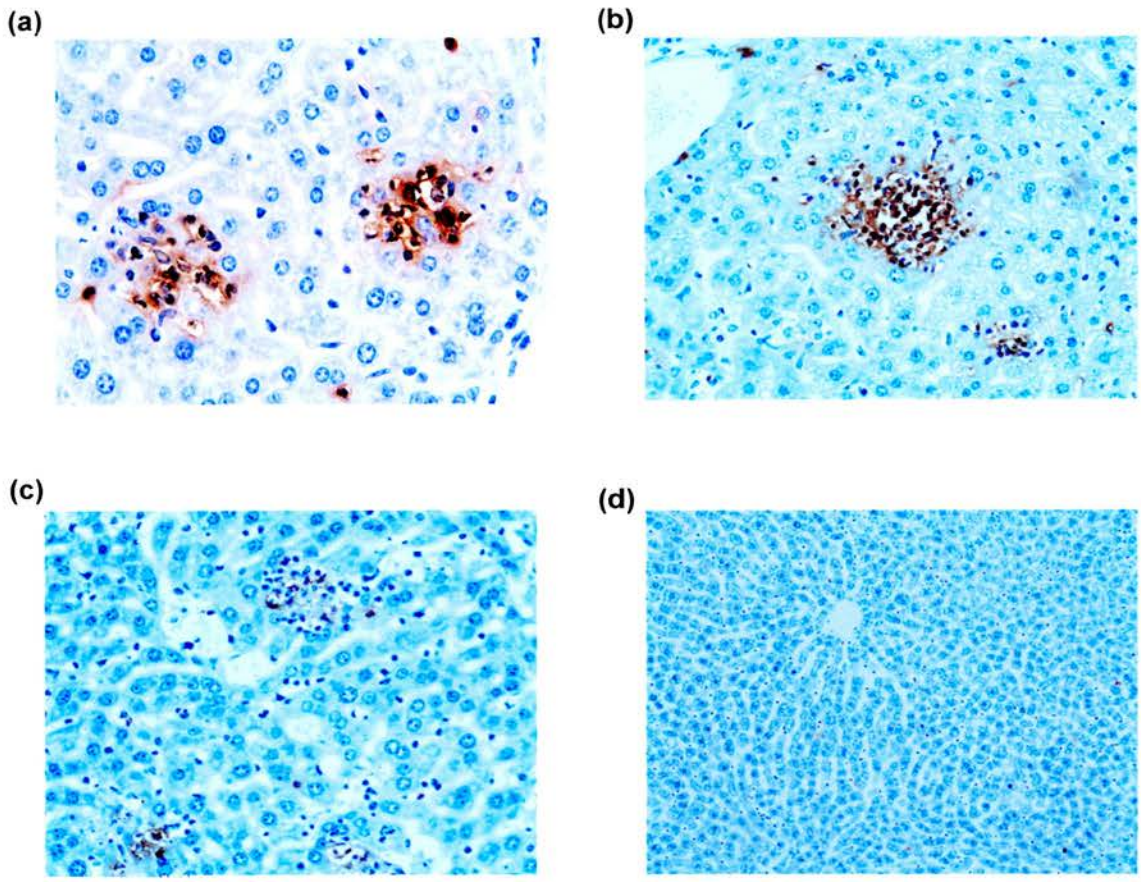


Figure 4.5 Detection of Ly-6G$^+$ cells in control and *C. abortus*-infected liver tissues. (a) Inflammatory foci in an infected mouse section (x400) (day 3 p.i.); **(b)** Large inflammatory foci in an infected mouse (x200) (day 5 p.i.); **(c)** One or two smaller inflammatory foci and circulating Ly-6G$^+$ cells (x200) (infected mouse, day 7 p.i.); **(d)** One or two circulating Ly-6G$^+$ cells in a control mouse section (x100) (day 6 p.i.).

Numbers of Ly-6G$^+$ cells were consistently higher in liver sections of pregnant than non-pregnant mice throughout the period of infection ($F_{1,13}$=5.68, p=0.041) (**Figure 4.6**). A significant decreasing trend in the number of Ly-6G$^+$ cells was observed in liver tissue of pregnant mice during the course of infection/pregnancy ($F_{3,11}$=3.95, p=0.047). No significant differences between time points were detected in non-pregnant mice during the infection ($F_{2,4}$=0.61, p=0.598), although a similar decreasing trend was observed from day 3 to 7 p.i.. Highest numbers of Ly-6G$^+$ cells were detected on day 3 p.i. after which numbers decreased in both pregnant and non-pregnant mice.

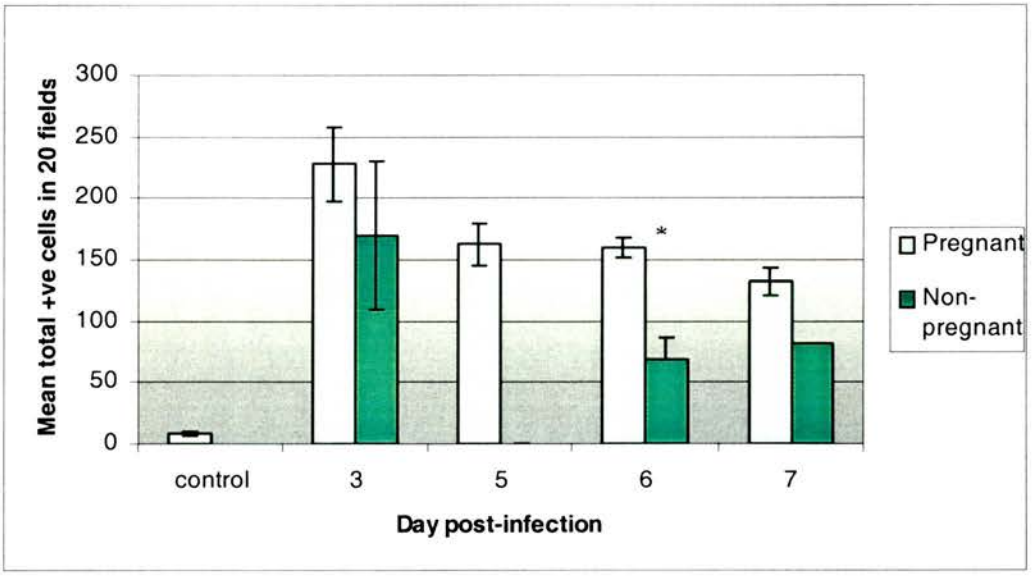


Figure 4.6 Number of Ly-6G$^+$ cells detected in liver sections from *C. abortus* infected pregnant and non-pregnant mice. Mean (± SEM) numbers of Ly-6G$^+$ cells in 20 fields of view (x20 magnification) were calculated in liver tissue collected from infected pregnant and non-pregnant mice at each time point. *Significant difference p<0.05 (ANOVA). Control group represents pooled data from all uninfected mice.

Ly-6G$^+$ inflammatory foci were more frequently observed than individual positive cells in placental tissue. Distribution of Ly-6G$^+$ cells in defined areas of placental sections are illustrated in **Figure 4.7**. As pregnancy and infection progressed, occasional inflammatory foci containing Ly-6G$^+$ positive cells were present in the labyrinth trophoblast cells (**Figure 4.7**), with inflammatory cells being detected in large numbers in all areas of placental tissue by day 7 p.i. (**Figure 4.7a,c**). A more

diffuse inflammatory infiltrate was detected in the maternal decidual cells, with only a few inflammatory foci being present (**Figure 4.7b**). Only small numbers of Ly-6G$^+$ cells were seen in sections from uninfected mice (data not shown).

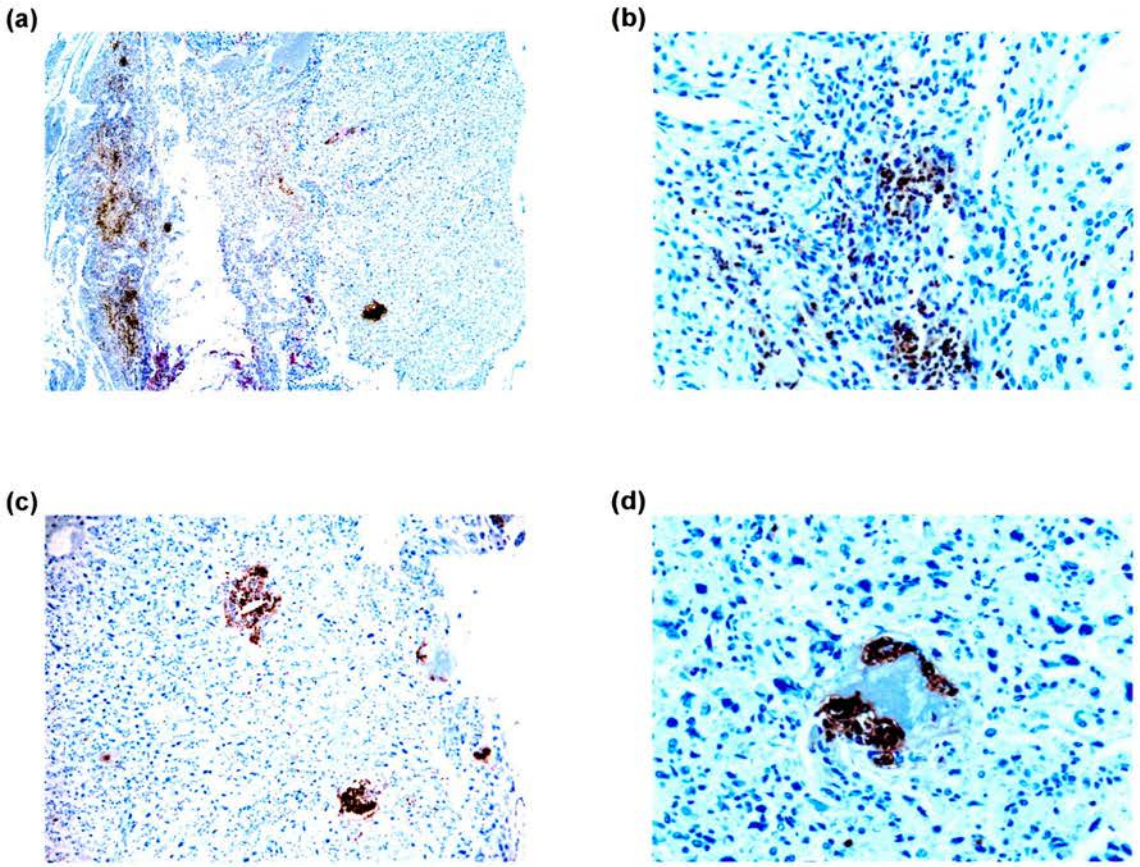


Figure 4.7 Detection of Ly-6G$^+$ cells in *C. abortus*-infected placental sections (from mice killed on day 7 p.i.). (a) Cross-section through placenta showing areas of Ly-6G labelling in the maternal decidua, giant cell layer and trophoblast cells (x100); **(b)** Ly-6G$^+$ cells in the maternal decidual area (x400); **(c)** Inflammatory foci in the labyrinth area at the foetal placental side (x200); **(d)** Inflammatory cells surrounding a chlamydial inclusion in trophoblast cells (x400).

Differences in Ly-6G$^+$ infiltrate in specific areas of the placenta over time are presented in **Figure 4.8** and are consistent with the kinetics of the appearance of chlamydial antigen during pregnancy (**Figure 4.4**). A slight increasing trend in numbers of Ly-6G$^+$ cells in the maternal decidua was observed as infection progressed, with the highest infiltrate detected on day 6 p.i. A similar trend was observed in the giant cell layer, with highest numbers of Ly-6G$^+$ cells detected on day 5 p.i. (**Figure 4.8**).

Highest numbers of Ly-6G$^+$ cells were observed in the foetal labyrinth area on day 6 p.i. No cells were detected in the uterine epithelium until day 7 p.i. with the exception of one on day 3 p.i. A clear increasing trend of Ly-6G$^+$ cell numbers in the maternal decidua was observed from day 3 to day 6 p.i., after which counts decreased. This is in contrast to liver tissue (**Figure 4.6**), where the inflammatory response decreased from day 3 p.i. in line with the decrease in the number of detectable *C. abortus* organisms.

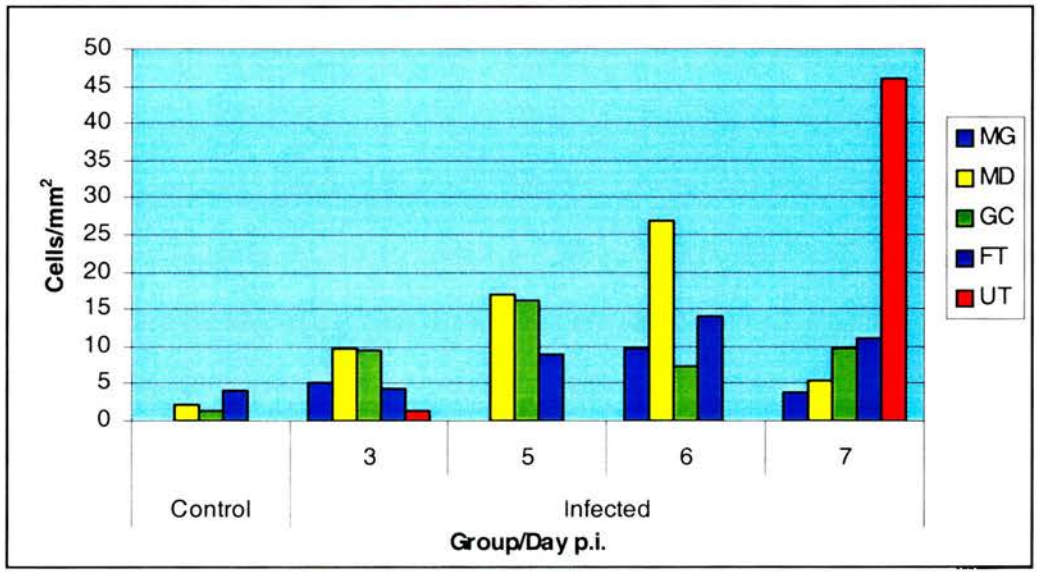


Figure 4.8 Numbers of Ly-6G$^+$cells/mm^2 in specific areas of uninfected and *C. abortus*-infected placental tissue during infection/pregnancy. MG= Metrial gland, MD= Maternal decidua, GC= Giant cell layer, FT= Foetal trophoblast cells, UT = Uterine epithelium. Control group represents pooled data from all negative control mice. NB. No metrial gland was present on day 5 p.i., so that no conclusions could be drawn regarding PMN infiltration into this area.

4.5.5.3 B cell detection in tissue sections

B cells, as revealed by expression of B220, were frequently seen both in inflammatory foci and individually, though more often in the latter state in liver tissue. Fewer B cells than PMNs were stained within inflammatory foci, as the majority of cells within these foci were Ly-6G$^+$, as described in the previous section (**Section 4.5.5.2**) and corroborated by other authors (Buendia *et al.,* 1998). **Figure 4.9** illustrates individual cells and small inflammatory foci of B220$^+$ cells, detected from days 3 to 7 p.i. Only occasional B cells were detected in liver sections from control mice (**Figure 4.9d**).

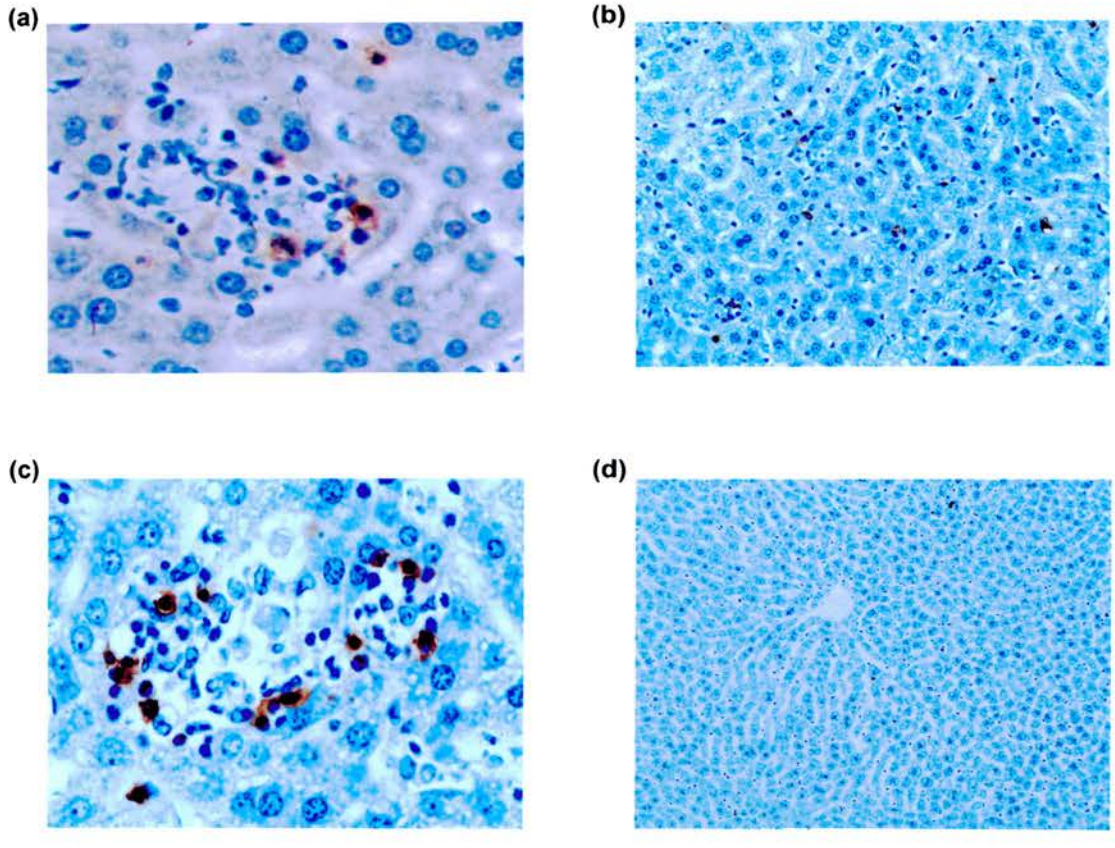


Figure 4.9 Detection of B220$^+$ cells in uninfected and *C. abortus*-infected liver sections. (a) Inflammatory focus and individual B220$^+$ cells in infected liver tissue day 3 p.i. (x400); **(b)** B220$^+$ cells in infected liver (day 5 p.i.) (x200) ; **(c)** Numerous B cells in an inflammatory focus in an infected liver section (day 7 p.i.) (x400); **(d)** Control liver section (day 6 p.i.) with very occasional B cells detected (x100).

Numbers of B cells counted in liver tissue sections are shown in **Figure 4.10**. B cell infiltration was clearly higher in infected than control mice during infection. Comparison of B cell numbers in liver sections from *C. abortus* infected pregnant and non-pregnant mice revealed significantly higher numbers in pregnant mice on day 6 p.i. only (ANOVA, $F_{1,3}$=11.14, p=0.044) (**Figure 4.10**), which was similar to the PMN response. There were no apparent differences in B cell numbers in liver sections from infected pregnant mice between time points during infection/pregnancy, with between 60-80 positive cells consistently detected in 20 fields of view ($F_{3,11}$=0.35, p=0.787). Similarly, there were no significant differences ($F_{2,4}$=0.12, p=0.894) in B cell numbers in liver sections from infected non-pregnant mice between time points.

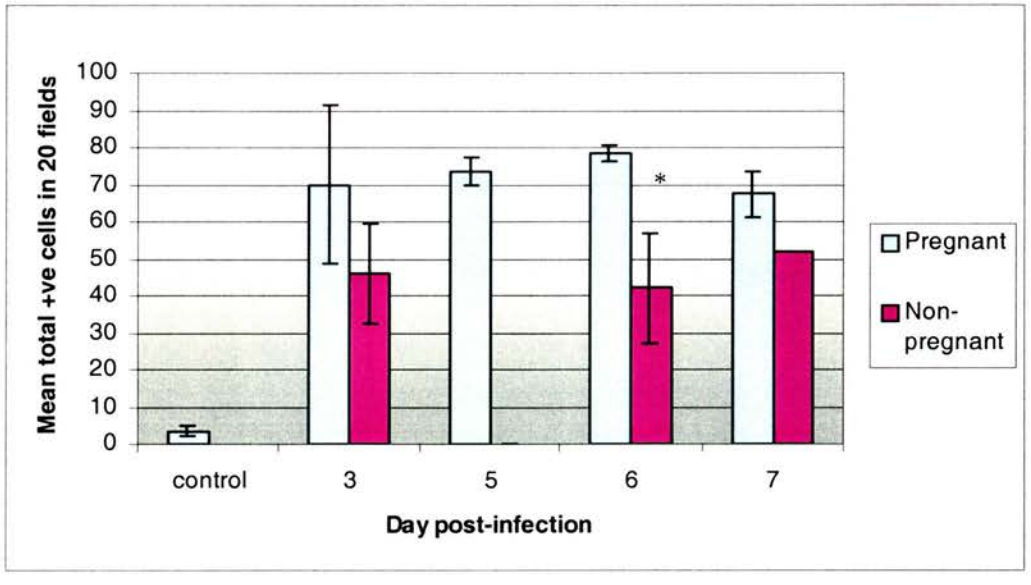


Figure 4.10 Numbers of B cells (B220$^+$cells) detected in liver tissue from *C. abortus*-infected mice. Mean (± SEM) B220$^+$ cells in 20 fields of view (x20 magnification) were counted from pregnant and non-pregnant mouse liver sections on each day p.i. *Significant difference p<0.05 (ANOVA). Control group represents pooled data from all uninfected mice. Note. No non-pregnant mice were sampled on day 5 p.i.

In placental tissue, the majority of B cells were observed in the maternal decidua area, with infrequent $B220^+$ cells observed in the labyrinth area, as illustrated in **Figure 4.11**. By day 7 p.i., B cells were rarely detected in the giant cell layer (**Figure 4.11b**) and $B220^+$ cells were only occasionally present in the labyrinth. In one infected mouse, $B220^+$ expression was absent even within the metrial gland and maternal decidua (**Figure 4.11d**).

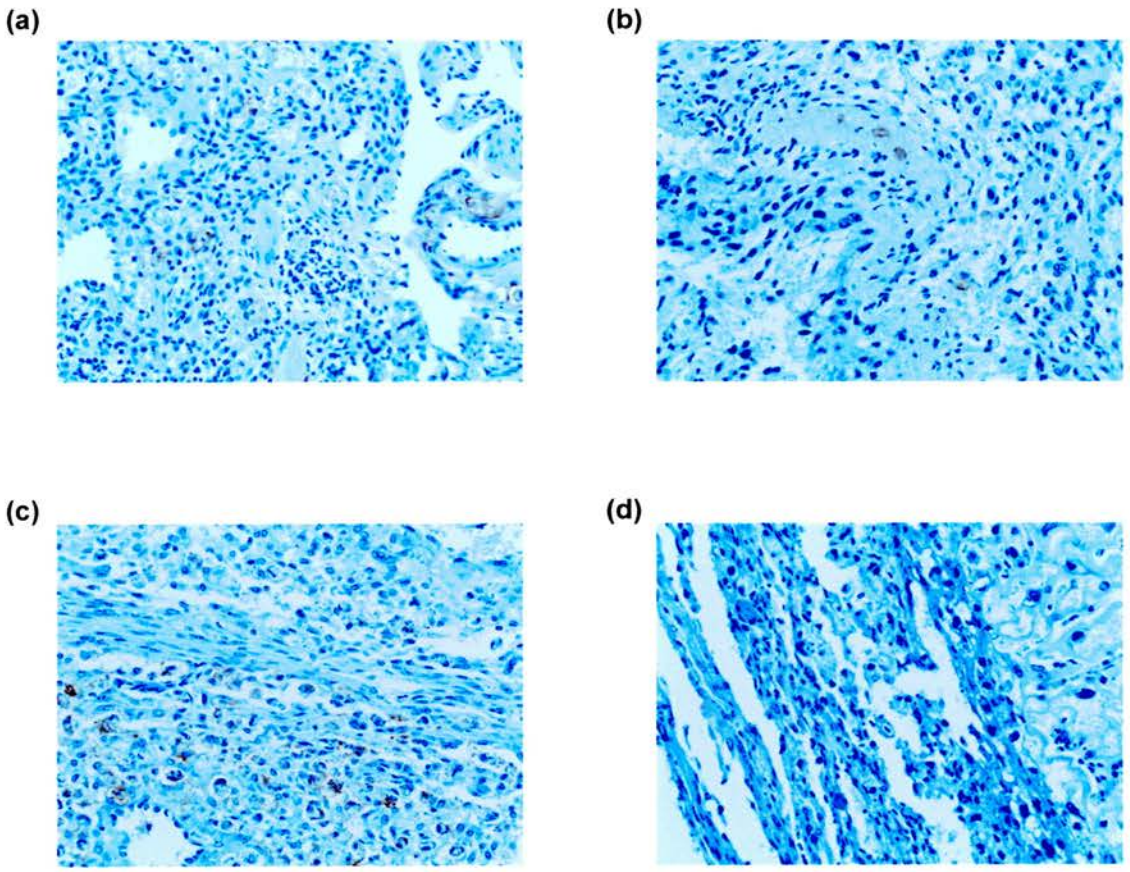


Figure 4.11 B cell detection in *C. abortus*-infected placenta sections. Detection of $B220^+$ cells in the maternal decidua at days 3 **(c)** (x400) and 7 p.i. **(a)** (x200) and **(b)** in the giant cell layer at day 7 p.i.(x400); **(d)** Maternal decidua from an infected mouse without B cells (day 5 p.i.) (x400).

Highest numbers of placental B220$^+$ cells were observed on day 3 p.i, in the metrial gland at the maternal side of the placenta (**Figure 4.12**). Counts were also noticeably high in the uterine epithelium on days 6 and 7 p.i. In general, B220$^+$ cell counts were lower than those of Ly-6G$^+$ cells throughout infection (**Figures 4.12** and **4.8**). Overall numbers of B cells increased in the placenta as infection progressed, being higher on days 6 and 7 p.i than days 3 and 5 p.i. Placentas of control animals were negative for B cells (**Figures 4.12**) despite the presence of low numbers of Ly-6G$^+$ cells within the same placental sections (**Figure 4.8**).

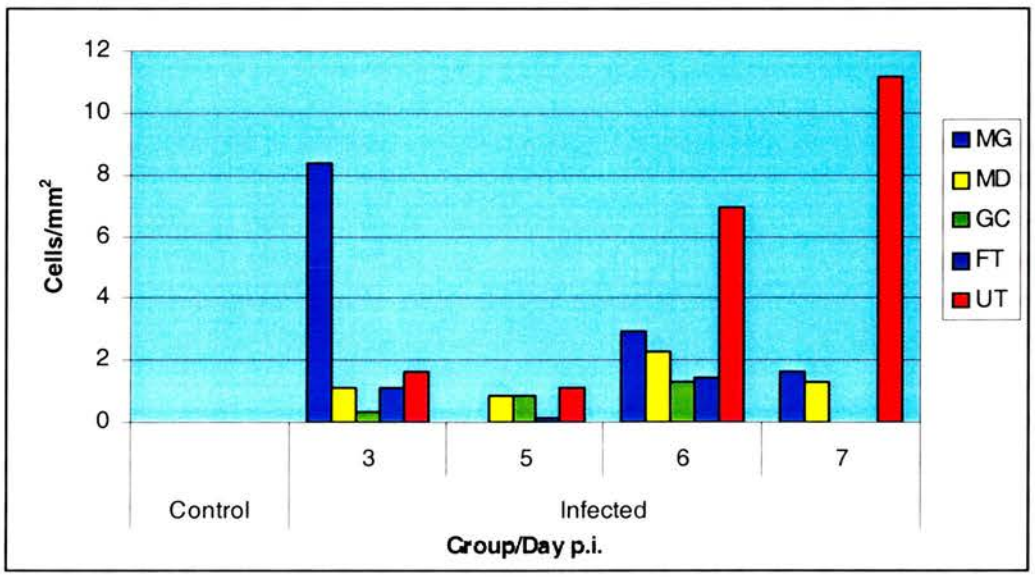


Figure 4.12 B220$^+$ cell counts in placenta tissue from control and *C. abortus*-infected mice. B220$^+$ cells were counted (x40 magnification) in each specific area of placenta on each day p.i. (cells/mm^2). MG= Metrial gland, MD= Maternal decidua, GC= Giant cell layer, FT= Foetal trophoblast cells, UT = Uterine epithelium. All placental tissue from control mice was negative for B220. NB. No metrial gland was present on day 5 p.i., so that no conclusions could be drawn regarding B cell infiltration into this area.

4.5.5.4 Comparison of PMN and B cell counts in *C. abortus*-infected liver sections

Numbers of Ly-6G$^+$ and B220$^+$ cells were counted in individual liver sections of pregnant mice to compare infiltration of the two cells types (**Figure 4.13**). **Figure 4.13** clearly shows that greater numbers of PMNs than B cells were recruited to sites of infection in liver tissue of pregnant mice ($F_{1,24}$=81.26, p<0.001) at all sample times during infection/pregnancy ($F_{3,24}$=3.29, p=0.045). Inflammatory foci were composed mainly of PMNs, with occasional B cells, which is consistent with a primary role for PMNs in controlling *C. abortus* infections. There were no significant differences between PMN and B cell counts in non-pregnant animals (data not shown) ($F_{1,10}$=2.30, p=0.130).

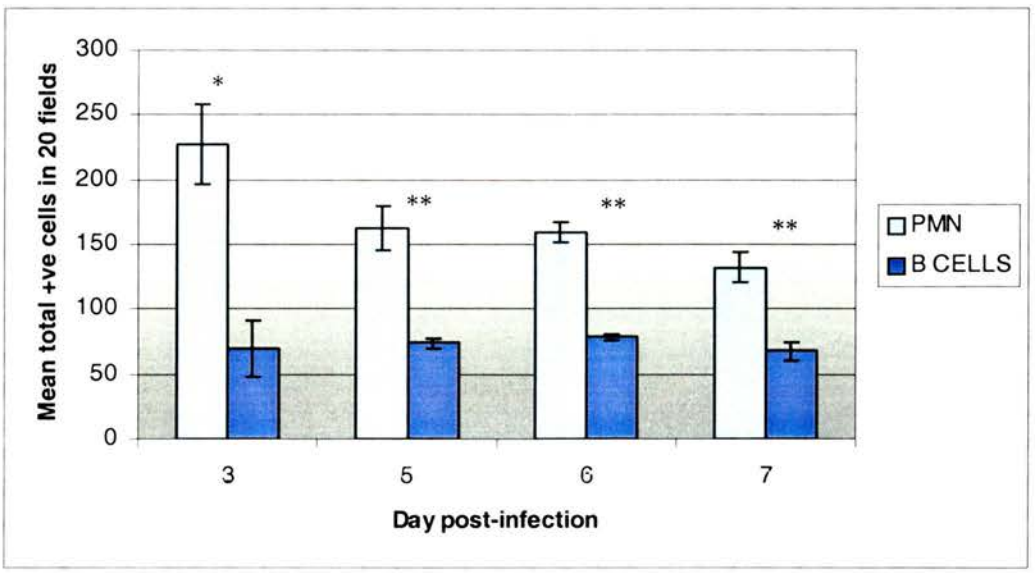


Figure 4.13 Comparison of B cell and PMN numbers in liver tissue of pregnant mice following infection with *C. abortus* on day 11 of pregnancy. Numbers of positive cells (mean ± SEM) were determined in 20 fields of view by light microscopy (x20 magnification) for each day p.i. Significant difference (ANOVA) **p<0.01, *p<0.05.

4.5.5.5 T cell immunohistochemistry

$CD3^+$, $CD4^+$ and $CD8^+$ T cell immunohistochemistry was attempted extensively without success in ZSF paraffin wax sections, as described in **Section 2.8.3**. Initial studies were conducted on *C. abortus*-infected spleen sections, as the spleen is rich in $CD4^+$ and $CD8^+$ T cells. Various antibodies from a range of companies were tested at different concentrations and varying conditions such as incubation times and temperatures. Despite extensive efforts, no positive cells were detected, even in spleen tissue. Chronically infected *Toxoplasma gondii* mouse tissues were also sampled to attempt to obtain positive control specimens but this was also unsuccessful.

4.5.6 Antibody isotyping

4.5.6.1 ELISA results

Isotypes of antibodies to *C. abortus* EBs were characterised in serum samples by ELISA (**Section 2.9.1**) to determine the nature of the immune response to infection in mice. ELISA results for IgG1, IgG2a and IgM isotypes are shown in **Figures 4.14, 4.15** and **4.16**, respectively.

Titres of IgG1, which is a dominant feature of Th2 immune response, were low throughout infection, with a significantly higher response ($F_{1,19}$=17.86, p<0.001) in serum from infected pregnant mice than in serum from control mice (**Figure 4.14**). The IgG1 response was highest on day 6 p.i., although there was no evidence of significant variations over the course of infection/pregnancy ($F_{3,19}$=1.69, p=0.218) in either control or infected pregnant mice. No significant differences were determined in IgG1 titres in non-pregnant mice on days 3, 6 or 7 p.i. ($F_{2,4}$=0.13, p=0.881). IgG1 titres in infected non-pregnant mice were similar to those of infected pregnant mice.

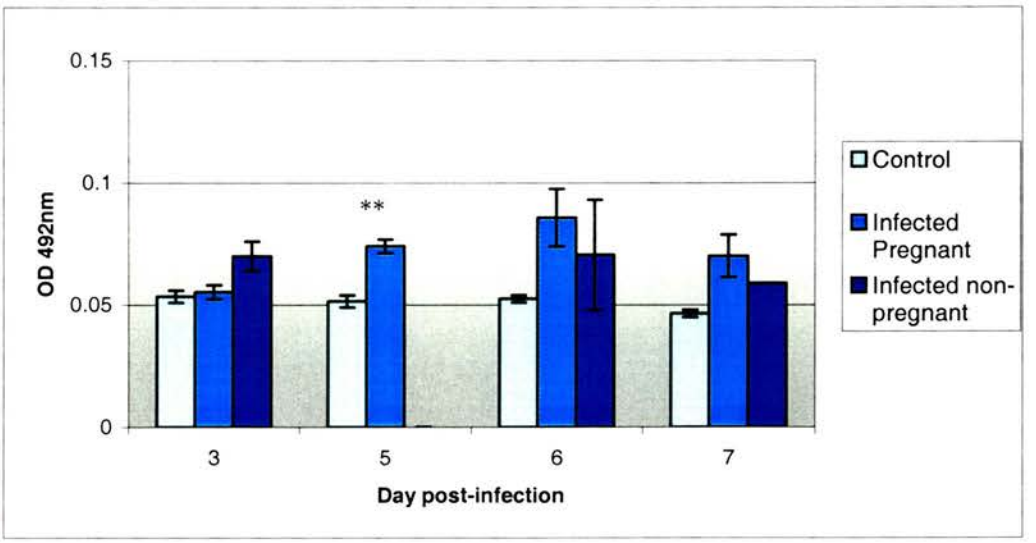


Figure 4.14 Serum IgG1 response to *C. abortus* infection in pregnant and non-pregnant mice. Mean ODs (± SEM) of IgG1 levels were determined on each day p.i. in sera from infected pregnant and non-pregnant and uninfected mice. ** Significant difference between control and infected mice p<0.01 (ANOVA). NB. No non-pregnant were sampled on day 5 p.i. and only one non-pregnant mouse was sampled on day 7 p.i.c

IgG2a responses in uninfected and *C. abortus*-infected mice were comparable on day 3 p.i. (**Figure 4.15**). However, IgG2a levels were higher in infected than control mice from day 5 p.i. onwards, and titres were highest on day 7 p.i. in serum from infected pregnant mice. Titres of IgG2a, which is indicative of a Th1 immune response in mice, increased significantly over the course of infection ($F_{3,11}$=11.75, p=0.002), with levels being higher on days 6 and 7 p.i. than on days 3 and 5 p.i.. In addition a significantly higher IgG2a response was detected in infected pregnant than control mice throughout infection/pregnancy ($F_{1,20}$=12.61, p=0.004). No significant differences were determined between IgG2a titres at different time points in *C. abortus*-infected non-pregnant mice ($F_{2,4}$=1.11, p=0.437), although highest titres were observed on day 6 p.i.. IgG2a titres in sera from infected pregnant mice were significantly higher to those observed in sera from infected non-pregnant mice ($F_{1,13}$=6.35, p=0.033).

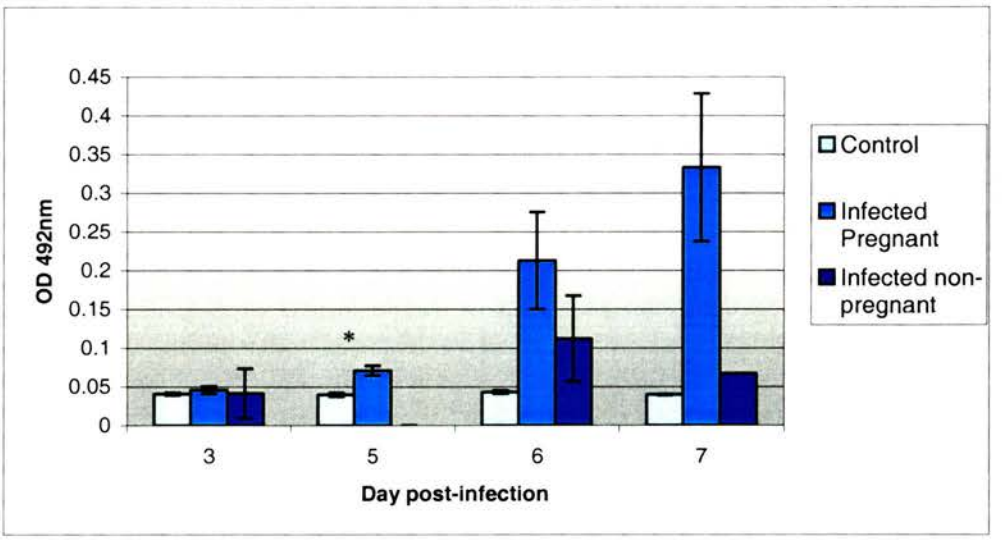


Figure 4.15 Serum IgG2a response to *C. abortus* infection in pregnant and non-pregnant mice. Mean ODs (± SEM) of IgG2a levels were determined in sera from uninfected and infected mice at different time points. *Significant difference between control and infected pregnant groups p<0.05 (ANOVA). NB. No non-pregnant were sampled on day 5 p.i. and only one non-pregnant mouse was sampled on day 7 p.i.

IgM titres were significantly higher in infected pregnant mice compared to control mice ($F_{1,19}$=81.32, p<0.001) throughout pregnancy/infection (**Figure 4.16**). IgM levels were significantly different during infection in pregnant mice sera ($F_{3,11}$=4.83, p=0.029), having increased on day 5 p.i. compared to day 3 p.i. and being highest on day 7 p.i.. In contrast, IgM responses did not significantly differ during infection in non-pregnant infected mice ($F_{2,4}$=0.28, p=0.773). No significant differences were determined between pregnant and non-pregnant *C. abortus*-infected mice ($F_{1,13}$=3.59, p=0.091).

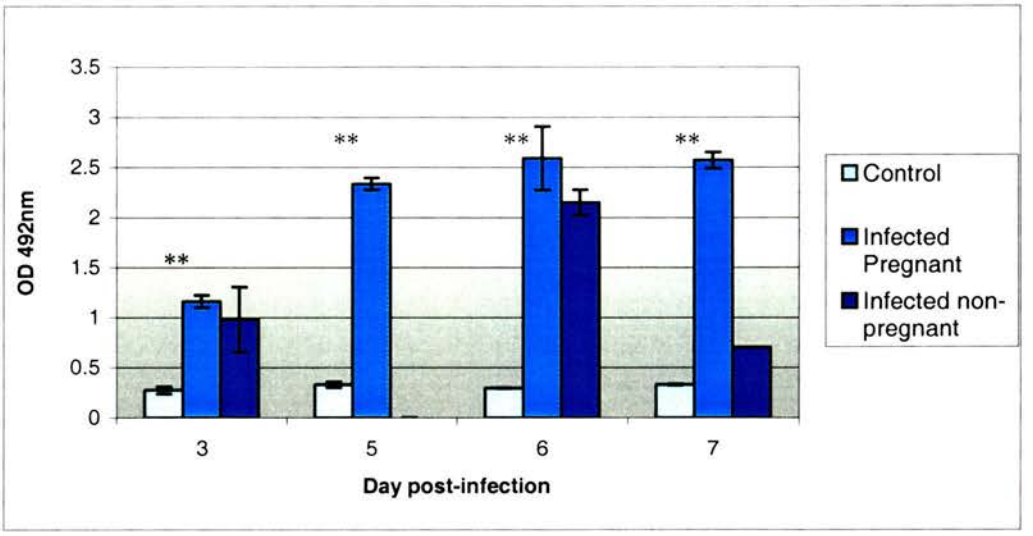


Figure 4.16 Serum IgM response to *C. abortus* infection in pregnant and non-pregnant mice. Mean ODs (± SEM) of IgM levels were determined in sera from uninfected and infected mice on at each time point. ** Significant difference between control and infected pregnant mice p<0.01 (ANOVA). NB. No non-pregnant mice were sampled on day 5 p.i. and only one non-pregnant mouse was sampled on day 7 p.i.

4.5.6.2 Western blotting

To determine the specificity of the antibody response, Western blotting analysis of whole EBs was conducted (**Section 2.9.2**). **Figure 4.17** shows IgG1 and IgG2a specificities of a range of sera from mice infected with *C. abortus* that were assessed using a multiscreen assay. In agreement with the ELISA data, an IgG1 response was undetectable in all mice except one, which was killed on day 7 p.i. (**Figure 4.17a**, lane 10). However, because the hyperimmune sera failed to show IgG1 reactivity it was difficult to conclude whether this was a true effect. It was not possible to repeat the analysis due to exhaustion of sera. Nonetheless, since ELISA titres also failed to increase in infected animals (**Figure 4.14**), it can be assumed that this was a genuine result.

In contrast, IgG2a antibodies revealed high specificity for EBs, with the positive control serum reacting to MOMP (approximately 38 kD) and to a range of proteins from 90 kDa to 55-60 kDa. Serum samples in lanes 4, 5, 6, 7 and 8 had strong reactivity to a range of proteins at approximately 55 kDa, and weaker reactivity at 21 kDa. Serum samples 4, 5, 6 and 7 were from mice killed on day 7 p.i., samples 8 and 9 from mice killed on day 6 p.i. and sera samples 10 and 11 were from mice killed on days 3 and 5 p.i., respectively. Serum samples in lanes 4 and 6 (day 7 p.i.) also produced a specific albeit weak MOMP response (38 kDa).

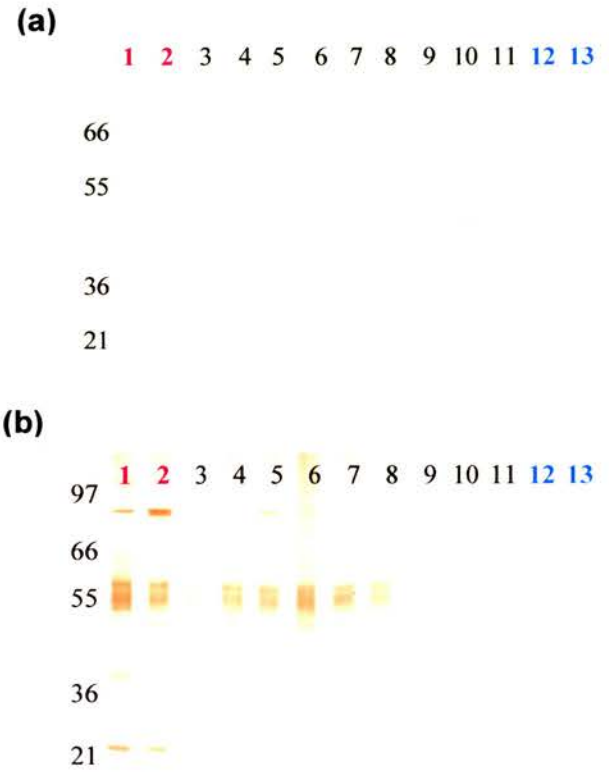


Figure 4.17 Western blots of control and *C. abortus*-infected mouse serum against whole EBs. Membranes were probed with sera from various mice, followed by either biotin conjugated goat anti-mouse IgG1 **(a)** or IgG2a **(b)** antibody and avidin peroxidase, and detected with DAB (**Section 2.9.2.3**). Samples **1** and **2** are positive controls of pooled convalescent sera from *C. abortus*-infected mice; **12** and **13** are negative controls; 3-11 infected mouse samples. Molecular masses (kDa) are indicated.

4.5.7 Cytokine ELISA

4.5.7.1 IFN-γ response to *C. abortus* infection in mice

A commercial ELISA was used to determine IFN-γ concentrations in serum samples collected at necropsy over the course of infection/pregnancy. **Figure 4.18** shows that IFN-γ concentrations in serum samples from infected mice were consistently higher than those in serum samples from control mice throughout infection. Serum IFN-γ concentrations showed an increasing trend as infection progressed, peaking at day 6 p.i., which was immediately prior to abortion. However this increase in cytokine levels over the course of infection/pregnancy was not significant ($F_{3,11}$=0.82, p=0.515). In contrast, cytokine levels in non-pregnant mice were significantly higher on day 3 than days 6 and 7 p.i. ($F_{2,3}$=98.71, p=0.002), coinciding with the reduction in bacterial burden in these non-pregnant animals. Concentrations of IFN-γ in sera of non-pregnant animals were less than those observed in pregnant animals (no significance, $F_{1,11}$=4.22, p=0.079). All serum samples from uninfected mice were negative.

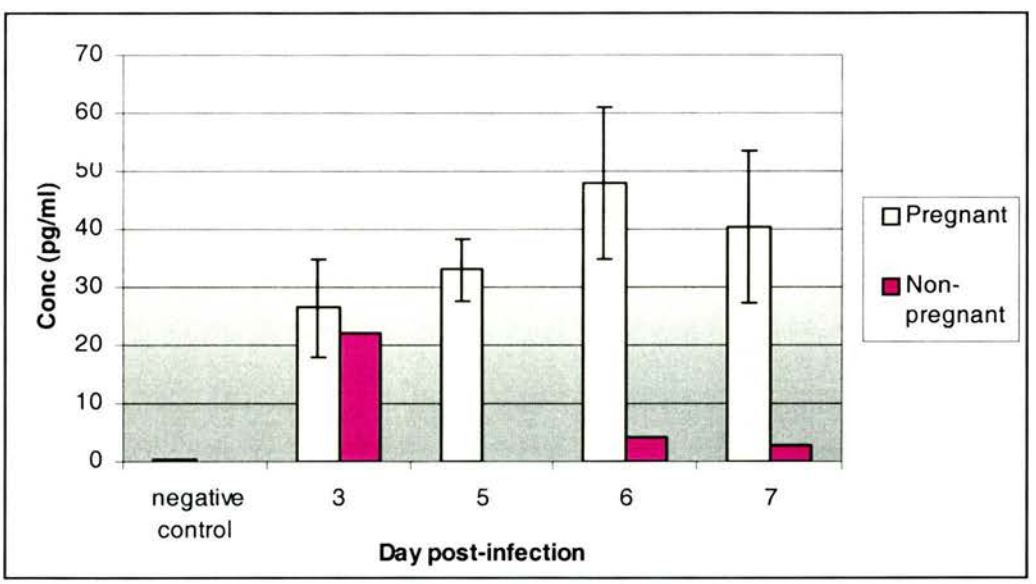


Figure 4.18 IFN-γ concentrations in sera from control and *C. abortus*-infected mice. Mean concentrations (± SEM) were determined for each group by extrapolation from a standard curve provided with the kit. The negative control bar represents pooled samples from control mice.

4.5.7.2 TNF-α response to *C. abortus* infection in mice

Serum concentrations of another important pro-inflammatory cytokine, TNF-α, during *C. abortus* infection of mice was estimated using a commercial ELISA. Serum TNF-α levels were considerably higher in infected pregnant mice than in infected non-pregnant and control mice, which with the exception of one animal, were negative (**Figure 4.19**). Levels of TNF-α expression showed an increasing trend from day 3 to day 7 p.i., with significant variations between the time points ($F_{3,11}$=17.45, p<0.001) (**Figure 4.19**). Cytokine levels in non-pregnant mouse sera were low with no significant differences observed throughout infection ($F_{2,2}$=0.14, p=0.881). Concentrations of TNF-α in *C. abortus*-infected non-pregnant mice were significantly lower than those in *C. abortus*-infected pregnant mice ($F_{1,11}$=39.0, p<0.001), although there was little difference between the groups on day 3 p.i., as demonstrated for the IFN-γ response. Both TNF-α and IFN-γ serum concentrations were highest at day 6-7 p.i., around the time of abortion.

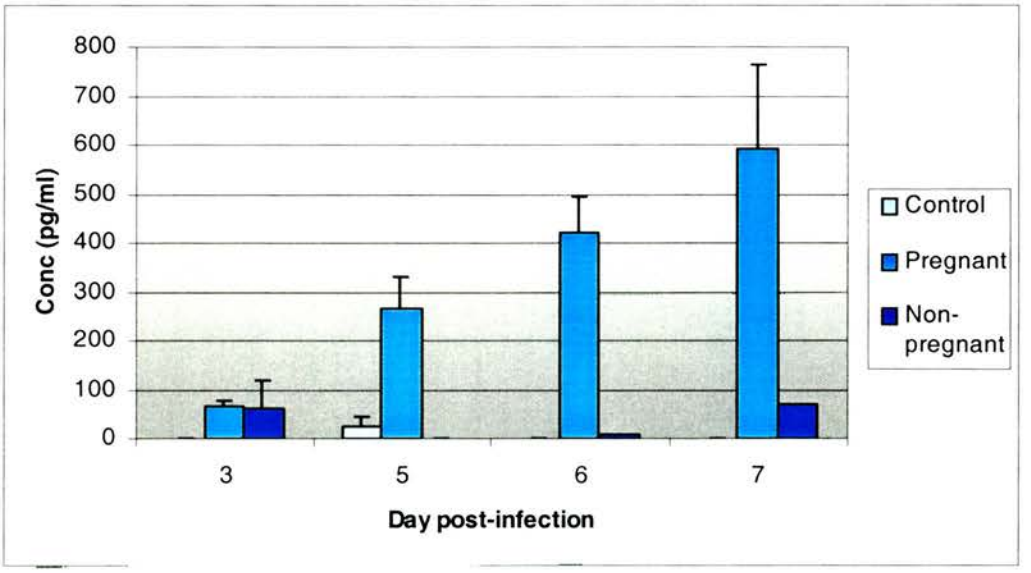


Figure 4.19 TNF-α concentrations in sera from control and *C. abortus*-infected mice. Mean concentrations (± SEM) were determined for each group by extrapolation from a standard curve provided with the kit. NB. No non-pregnant mice were sampled on day 5 p.i.

4.5.8 *In situ* hybridisation

4.5.8.1 Detection of IFN-γ mRNA$^+$ cells

Expression of IFN-γ in tissues was determined by *in situ* hybridisation using specific DIG-labelled probes (**Section 2.11**). Positive cells, detected using a DIG-labelled anti-sense IFN-γ probe, in liver and placenta are shown in **Figure 4.20**, alongside a section probed with a DIG-labelled sense IFN-γ probe (**Section 2.11.5**). IFN-γ mRNA$^+$ cells were generally associated with sites of inflammatory cell foci in liver sections (**Figure 4.20b**), where *C. abortus* antigen was routinely detected (**Chapter 3, Figure 3.6b**). Isolated individual mRNA$^+$ cells were also present, although these were less common. In contrast, positive cells were far fewer in number and distribution in placenta and were generally observed as single positive cells in the uterine epithelium, with only occasional IFN-γ mRNA$^+$ cells present within the decidual and trophoblast cell areas (**Figure 4.20d**).

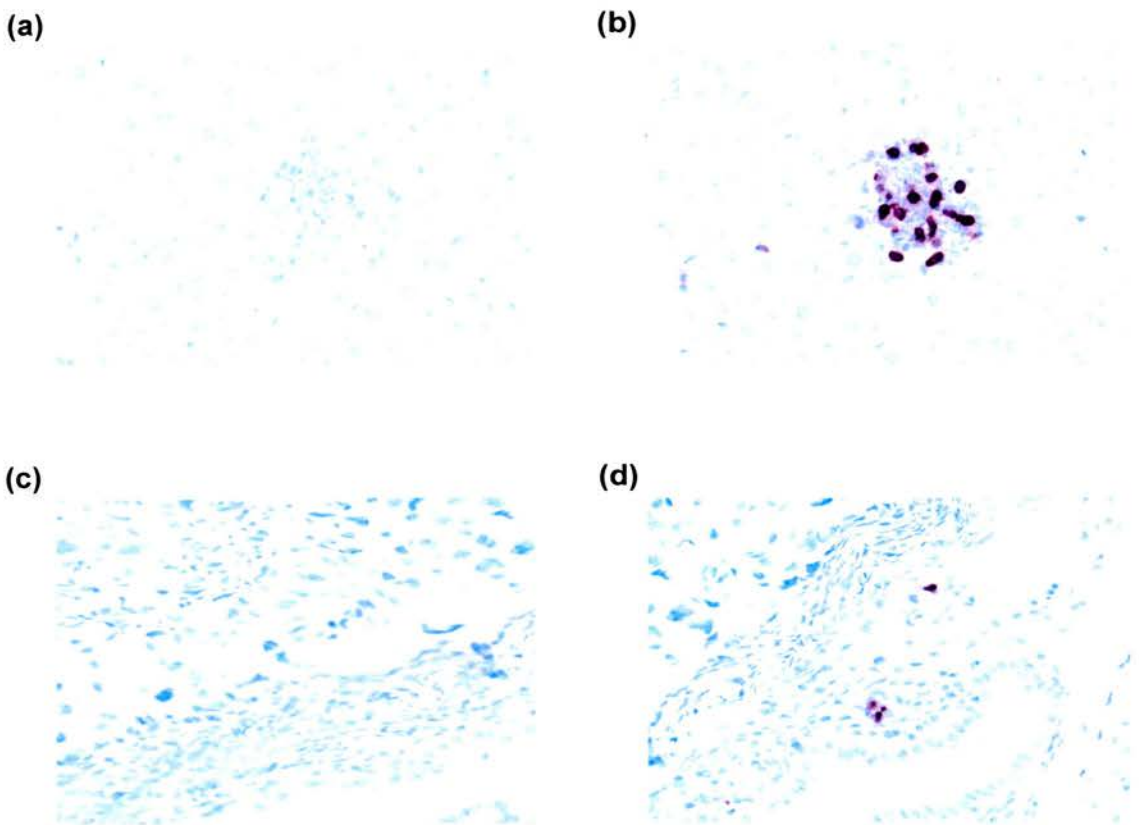


Figure 4.20 IFN-γ mRNA$^+$ cells in *C. abortus*-infected tissue sections. Liver tissue (x200) labelled with **(a)** control sense (T7) probe **(b)** anti-sense (SP6) probe; Uteroplacental tissue labelled with **(c)** control sense (T7) probe (x200) and **(d)** anti-sense (SP6) probe (x100).

To evaluate the influence of pregnancy on expression of IFN-γ in liver during the course of infection numbers of positive cells were counted (**Figure 4.21**). There were significant differences in IFN-γ mRNA expression during infection in pregnant ($F_{3,11}$=4.54, p=0.039) but not in non-pregnant mice ($F_{2,4}$=0.24, p=0.798). In a similar manner to serum IFN-γ concentration, there was an increasing trend as infection progressed, with a peak on day 6 p.i. IFN-γ mRNA response was significantly ($F_{1,13}$=50.78, p<0.001) lower in infected non-pregnant than infected pregnant mice liver sections throughout infection.

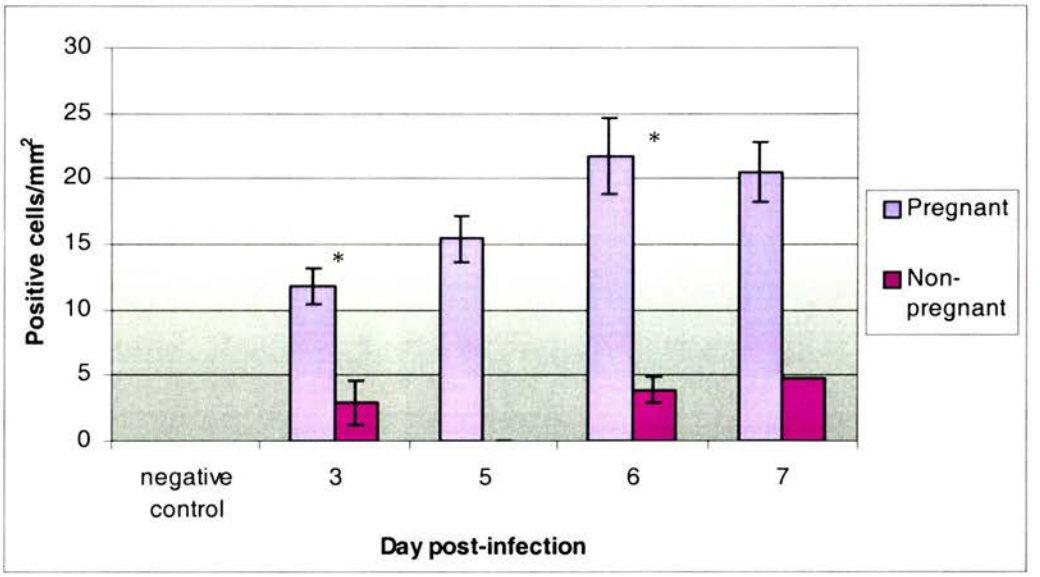


Figure 4.21 Numbers of IFN-γ mRNA$^+$ cells in *C. abortus*-infected liver tissue. Numbers of positive cells were counted (x40 magnification), expressed as the mean (± SEM) number of positive cells/mm^2 for each day p.i. *Significant difference p<0.05 (ANOVA) between pregnant and non-pregnant mice. The negative control bar represents pooled samples from control mice. NB. No non-pregnant mice were sampled on day 5 p.i. and only one non-pregnant mouse was sampled on day 7 p.i.

IFN-γ mRNA$^+$ cells were fewer in placental than liver tissue and were generally restricted to the maternal areas, although occasional positive cells were detected in the foetal labyrinth (**Figure 4.22**). Positive cells were also detected in the uterus, although in smaller numbers than in liver tissue **(Figure 4.22).** Numbers of IFN-γ mRNA$^+$ cells detected in uteri on day 6 p.i. were clearly higher than those detected

on day 3 p.i.. However, in placenta tissue, huge variation was observed in mice sampled on day 5 p.i., ranging from approximately 1.45 cells/mm^2 to 7.5 cells/mm^2. Not all infected placental tissues sections that were analysed contained positive cells and all uninfected mice were negative for IFN-γ mRNA.

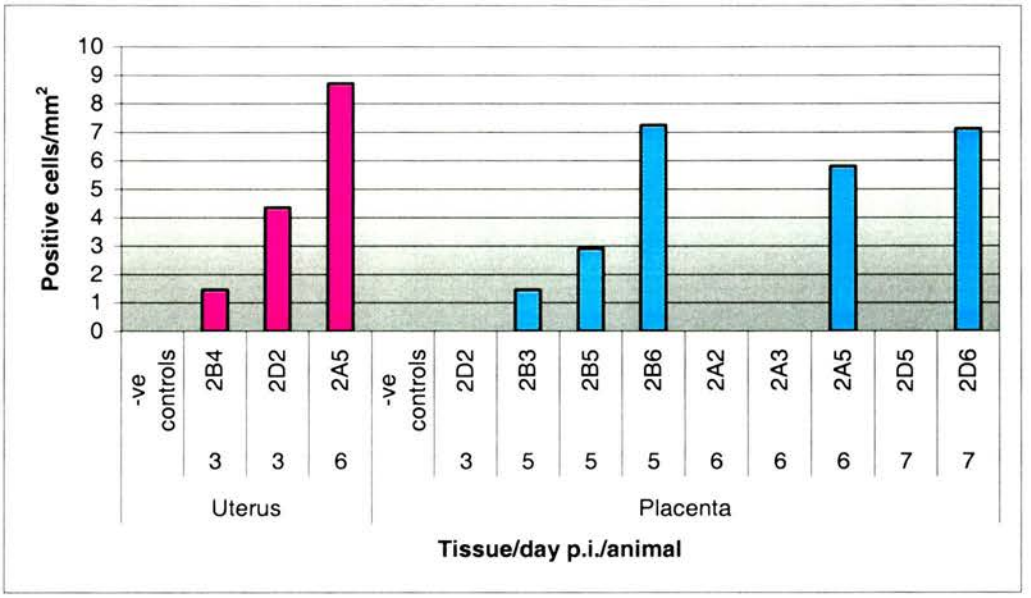


Figure 4.22 IFN-γ$^+$ cell counts in control and *C. abortus*-infected uterine and placental tissue. Numbers of positive cells counted per mm^2 (x40 magnification) are shown for each pregnant animal. Control groups represent pooled data from all negative control mice.

4.5.8.2 Detection of TNF-α mRNA$^+$ cells

In general, numbers of TNF-α mRNA$^+$ cells were low throughout infection in comparison to those expressing IFN-γ mRNA (**Figure 4.20**). Representative images of positive cells in liver tissue are shown in **Figure 4.23**. TNF-α mRNA$^+$ cells were occasionally present in inflammatory infiltrates (**Figure 4.23b**) but were routinely identified as single positive cells scattered throughout the tissue sections (**Figure 4.23d**). All uninfected mouse liver sections were negative for TNF-α mRNA expressing cells.

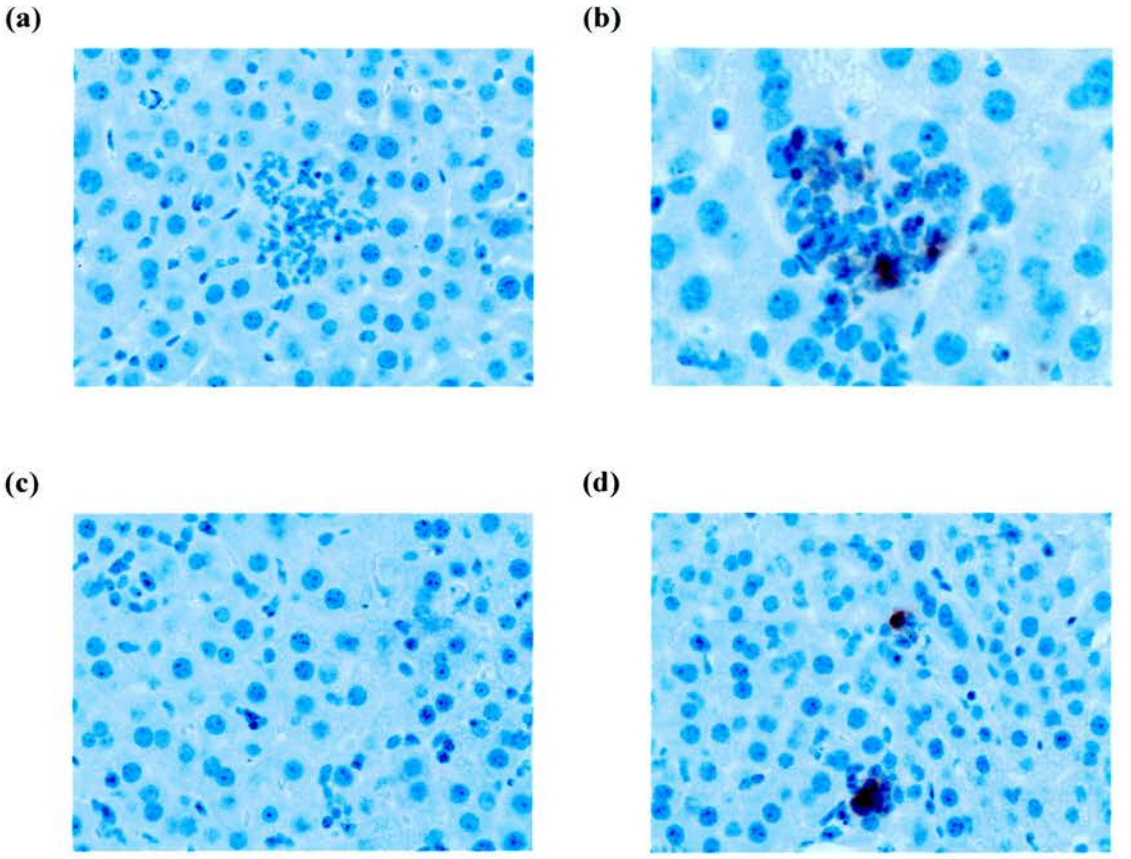


Figure 4.23 TNF-α mRNA detection in *C. abortus*-infected liver tissue from mice killed on day 6 p.i. (a) & (c) Labelled with control sense (T7) probe (x400); **(b)** (x600) **& (d)** (x400) Labelled with anti-sense (SP6) probe.

Numbers of TNF-α mRNA expressing cells in both liver (**Figure 4.24**) and placenta were lower than those observed for IFN-γ (10-20 fold less, except on day 6 p.i.), in contrast with ELISA results (**Section 4.5.7.2**). Highest numbers of TNF-α cells were detected in pregnant and non-pregnant mice livers on day 6 p.i., and the number of TNF-α^+ cells was significantly higher in pregnant than non-pregnant mice throughout the infection ($F_{1,13}$=11.66, p=0.008). Significantly fewer positive cells were present in liver tissue from the infected pregnant mouse group on days 3, 5 and 7 p.i than the on day 6 p.i. ($F_{3,11}$=13.16, p=0.001), which is immediately prior to or at the time when abortions occurred.

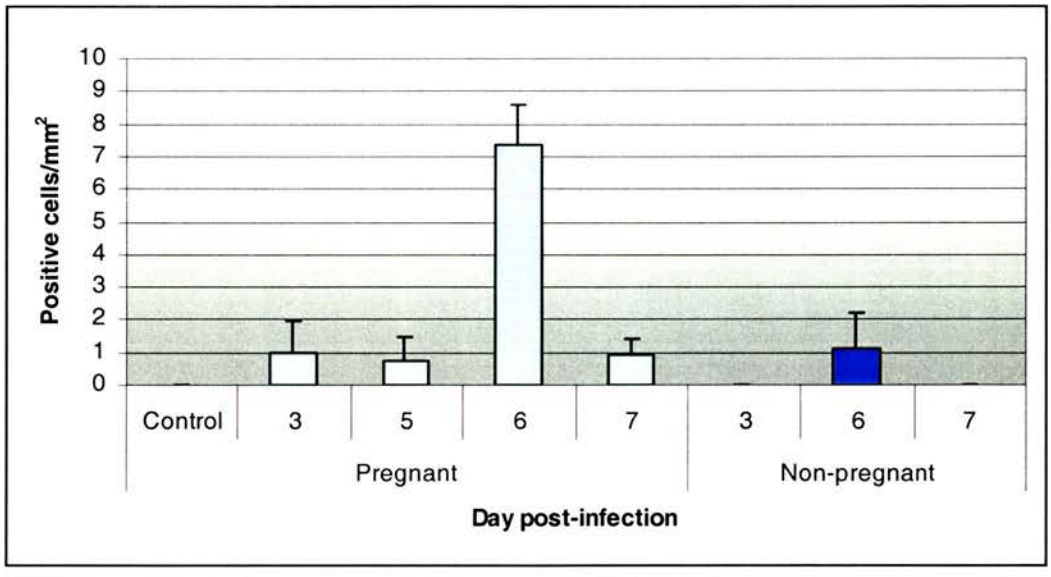


Figure 4.24 TNF-α cell counts in liver tissue from *C. abortus*-infected pregnant and non-pregnant mice. Numbers of positive cells were counted, expressed as the number of positive cells/mm^2 for each group. Control group represents pooled data from all negative control mice.

In contrast to IFN-γ mRNA$^+$ cell counts, TNF-α mRNA$^+$ cells were detected in placentas of only two mice, one of which was killed on day 6 p.i. with 1.45 cells/mm^2, and the other on day 7 p.i. with 4.35 cells/mm^2. Numbers of TNF-α mRNA$^+$ cells were higher in liver than placental tissue on day 6 p.i., whereas on day 7 p.i. counts in placenta were clearly higher than those in liver tissue. Very occasional TNF-α mRNA$^+$ cells were identified within the foetal labyrinth, with the remainder being localised in maternal tissue.

4.6 Discussion

Characterisation of the immune response to *C. abortus* in this CBA mouse model was necessary to validate its use in future studies and this study complemented previous analyses carried out in *C. abortus*-infected mice (Buendia *et al.*, 1998), 1999), where several features of a Th1 response were identified (Buxton *et al.*, 2002).

The inbred CBA mouse strain was chosen over the outbred Porton strain to allow comparison and statistical analysis of immune responses between genetically identical mice. As was clearly demonstrated in the pilot study, *C. abortus* gave rise to a systemic infection that targeted the liver and placenta and resulted in abortion. The pregnancy rate was notably better in this study than in the pilot experiment, as a result of the synchronising Whitten effect, which was then adopted in subsequent matings.

In terms of the recovery of *C. abortus*, numbers of organisms were significantly higher in placenta than those in liver throughout infection/pregnancy. This was consistent with the placenta being the target organ for *C. abortus* in infected pregnant mice (Buendia *et al.*, 1998; Buzoni-Gatel *et al.*, 1992) (**Chapter 3**) and sheep (Buxton *et al.*, 1990; Stamp *et al.*, 1950). In accordance with the pilot study, no significant differences were detected over the course of infection/pregnancy in the titres of *C. abortus* in liver or placenta. However, chlamydial antigen counts showed an increasing trend in both tissue types as infection and pregnancy progressed. In agreement with recovery data, numbers of chlamydial organisms detected by immunohistochemistry were significantly higher in placenta than liver tissue, reflecting progression of *C. abortus* from peripheral tissues to the target organ. Non-pregnant mice showed extremely low levels of antigen, illustrating the difference in susceptibility between pregnant and non-pregnant animals, which may reflect the immunosuppressed nature of pregnant mice, as previously discussed in **Chapter 3**.

Ly-6G$^+$ and B cell numbers were higher in liver tissue of pregnant than non-pregnant mice, possibly due to the overall higher levels of infection in the former, as demonstrated by the greater number of detectable organisms. The influx of Ly-6G$^+$ in liver was highest on day 3 p.i. and gradually decreased thereafter in both pregnant and non-pregnant mice. This was perhaps due to movement of the organisms to the placenta in pregnant mice and to the earlier clearance of *C. abortus* from livers of non-pregnant mice. As observed by Buendia *et al.* (1999) greater hepatic damage was observed in pregnant than non-pregnant mice, with numerous foci of PMN and mononuclear cells. In contrast, B cell numbers did not vary significantly during infection and differences between pregnant and non-pregnant animals were not as pronounced as those observed for PMNs. Comparison of PMN and B cell infiltrates into the liver tissue of pregnant mice showed significantly higher numbers of PMNs than B cells throughout infection. This concurs with other mouse model experiments, in which the essential role of PMN in primary infections and the minimal role of B cells in early infections have been demonstrated (Buendia *et al.,* 1999, 2002). The decrease in PMN infiltration with progression of infection is likely to be associated with their ability to recruit other cell types to the sites of infection, thus diminishing their role as specific immunity starts to develop (Romani *et al.*, 1997).

Although PMN and occasional B cells were identified in inflammatory foci in liver and maternal placental areas, it is probable that monocytes and T cells, which are present in the inflammatory exudates in infected ovine organs, (Navarro *et al.*, 2004) were also present. Circulating (individual) PMNs and B cells were frequently observed within liver sections and were also present in large numbers in spleen sections. No areas of necrosis, which are common in mice that have been depleted of PMNs (Buendia *et al.,* 1999), were observed in liver or spleen. In placenta, PMN and B cell infiltration was more extensive in maternal than foetal areas, despite the high numbers of infectious organisms present in the foetal labyrinth. Although occasional large inflammatory foci consisting of granulocytes were detected in the foetal labyrinth, often surrounding chlamydial inclusions as infection progressed.

Antibody alone is not correlated with protection, but in the mouse, it has been suggested that IgG1 is associated with a Th2 immune response and IgG2a with a Th1 immune response (Snapper & Paul, 1987; Stevens *et al.*, 1988) and it is well documented that a Th1 response is necessary to control *Chlamydia* infections (Buzoni-Gatel *et al.*, 1992; Igietseme *et al.*, 1993). Therefore, by identifying antibody isotypes and cytokines produced in response to *C. abortus* infection, the nature of the immune response can be determined. A low IgG1 response to *C. abortus* EBs was detected throughout infection in CBA mice, whereas a marked increase in the IgG2a response occurred towards the latter stage of infection. This is consistent with a Th1 type immune response in this model, and is comparable to responses detected post-*C. trachomatis* infection in mice (Igietseme *et al.*, 1998).

IgG2a levels were similar to those of uninfected mice on day 3 p.i., whereas by days 5, 6 and 7 p.i., infected pregnant mice had a higher IgG2a response than control mice. IgM levels were higher in sera of infected mice than that of control mice, showing that the primary antibody response had developed in response to *C. abortus* infection. It would be interesting to monitor the IgG2a response post-abortion to determine if it is long-lived as in sheep, where seroconversion occurs immediately prior to abortion (Longbottom & Coulter, 2003). This was beyond the scope of the present study. Western blotting further verified the specificity of the IgG2a antibody response to *C. abortus* EBs, with reactivity against a range of proteins.

The role of inflammatory cytokines, IFN-γ and TNF-α, is well documented in all *Chlamydia* infections and both are highly characteristic of the controlling Th1 type immune response. Levels of these cytokines in sera were determined to investigate whether *C. abortus* infection in this mouse model would induce similar responses to those observed in sheep and to other species of *Chlamydia*. No IFN-γ was detected in sera from non-infected mice, whereas 25-50 pg/ml of IFN-γ was found in sera from infected pregnant mice over the course of pregnancy/infection, indicating that the cytokine was produced in response to *C. abortus* infection. Highest concentrations were observed on day 6 p.i., immediately prior to abortion, suggesting that IFN-γ may contribute to the induction of abortion. The release of IFN-γ by inflammatory

cells (such as macrophages and T cells) leads to further recruitment of inflammatory cells to sites of infection. This could induce pathological damage, resulting in impaired placental function and abortion, rather than control of the infection. However, since IFN-γ primarily controls *C. abortus* and other *Chlamydia* infections *in vitro* (Brown & Entrican, 1996; Graham *et al.*, 1995; Summersgill *et al.*, 1995) its precise role *in vivo* is unclear. The observation of highest IFN-γ concentrations around the time of abortion is consistent with a role for IFN-γ in control of the infection at this time, as its production was increased when high numbers *C. abortus* organisms were detected.

Other *C. abortus* models have yielded much higher levels of IFN-γ in serum, averaging 6000 pg/ml from day 2 p.i. onwards (Buendia *et al.*, 2004) than those observed in this study. These differences could be related to the more resistant C57BL/6 mouse strain infected with *C. abortus* by Buendia *et al.* (2004) in their study. Infection of C57BL/6 mice has previously been shown to result in faster clearance of *C. abortus* infection from these mice than that seen with CBA mice (Del Rio *et al.*, 2000). These differences may also have arisen from the use of different ELISA kits with distinct sensitivities.

Kinetics of the appearance of IFN-γ mRNA$^+$ cells in the tissues were similar to those observed in serum, with a peak in the number of cells on day 6 p.i.. Considerably more IFN-γ mRNA$^+$ cells were present in liver sections than in placenta of infected mice and the cytokine was detected within cytoplasm of inflammatory cells in the liver, suggesting production by macrophages, T cells or granulocytes (Ellis & Beaman, 2004; Rothfuchs *et al.*, 2004; Rottenberg *et al.*, 2002). It also appeared that IFN-γ mRNA production was localised to sites where chlamydial antigen was detected in serial liver and placental sections. Double staining of IFN-γ mRNA by *in situ* hybridisation followed by immunohistochemistry to detect chlamydial antigen was attempted unsuccessfully on single sections. However, by conducting *in situ* hybridisation and immunohistochemistry on serial sections, IFN-γ mRNA$^+$ cells were observed to colocalise with *Chlamydia* antigen and inflammatory foci, suggesting release of IFN-γ by PMNs and a role for IFN-γ in controlling *C. abortus*

infections in this mouse model. IFN-γ mRNA$^+$ cells were few in number in placental tissues and distribution was generally within the uterine epithelium, where the majority of the inflammatory infiltrate was detected, although occasional IFN-γ mRNA$^+$ cells were detected among maternal decidual and foetal trophoblast cells. This is in agreement with the few IFN-γ mRNA encoding cells identified in sheep studies, which were associated with or adjacent to areas of arteritis (Buxton *et al.*, 2002), but contradicts the theory of a role for IFN-γ in control in infected placenta (Brown & Entrican, 1996).

NK cells have been associated with IFN-γ production and control of infection for species such as *Plasmodium* (De Souza *et al.*, 1997) and *Listeria* (Dunn & North, 1991). IFN-γ can also be produced by macrophages, as reported for a related species *C. pneumoniae* (Rottenberg *et al.*, 2000). Although NK cell and macrophages were not examined in this experiment, it would be extremely interesting to evaluate these cell populations in future experiments to identify the cells that produce IFN-γ in *C. abortus* infections. Buendia and others (2004) reported that depletion of NK cells led to uncontrolled multiplication of *C. abortus*, suggesting that IFN-γ production was diminished in the absence of NK cells. They observed an association between IFN-γ and NK cells on day 4 p.i., although the cytokine was detected in the absence of NK cells on day 2 p.i. IFN-γ treatment of PMNs has been shown to enhance killing of bacteria such as *Brucella abortus* and *Mycobacterium tuberculosis* (Ellis and Beaman, 2004) by various mechanisms. It would be useful to examine this in *C. abortus* infections given the association between IFN-γ expression and PMN infiltration observed in this and other studies.

Although TNF-α mRNA expression was readily detected in the cytoplasm of mononuclear cells in infected ovine placentas (Buxton *et al.*, 2002), few cells expressing TNF-α mRNA were detected in the liver or placenta of mice in this study, despite the high concentration of this cytokine in sera from infected mice (80 – 600 pg/ml from days 3 to 7 p.i., respectively). In this study, serum TNF-α concentrations in infected mice were remarkably higher than IFN-γ concentrations detected. TNF-α may play either a role in either the control of the infection as an increasing trend in

TNF-α concentrations was observed as infection progressed, or alternatively, this cytokine may play a role in abortion as it has been linked to abortion and miscarriage (Arslan *et al.*, 2004; Erlebacher *et al.*, 2004; Soto *et al.*, 2003). The higher TNF-α concentrations on day 6-7 p.i. at the time of abortion may therefore be of significance, even though the cytokine was not expressed at localised sites of infection in the placenta. TNF-α, which is produced in response to *T. gondii* infections, can have deleterious effects on oocyte maturation that compromises the development of the resultant embryo in cattle (Soto *et al.*, 2003), suggesting a role for TNF-α in induction of abortion in *C. abortus*-infected sheep (Buxton *et al.*, 2002) but not mice. Differences such as the length of pregnancy and immune responses in sheep and mice will ultimately lead to differences in control of *C. abortus* infections and in the induction of abortion. Although inflammatory responses are observed both in mice and sheep, the longer pregnancy in sheep allows more extensive pathological damage and necrosis to develop compared to that in mice and this may be related to the higher expression of TNF-α in sheep placenta than in mice placenta and contribute to abortion.

Like IFN-γ, TNF-α mRNA was expressed in cells associated with inflammatory foci and it is known that TNF-α can be released by PMN (Schaller *et al.*, 2004). In contrast, Buendia *et al.* (1999) described high TNF-α expression in *C. abortus*-infected PMN-depleted mice. In this study the numbers of TNF-α mRNA$^+$ cells in the cytoplasm of cells in liver and placenta were substantially lower than numbers of IFN-γ mRNA$^+$ cells. Highest numbers of TNF-α$^+$ cells were detected on day 6 p.i. in the liver, which coincides with the pre-abortion and the high serum concentration of TNF-α on day 6 p.i.. TNF-α mRNA expressing cells were detected in only two of the placentas tested, in marked contrast to what is observed in infected ovine placentas (Buxton *et al.*, 2002), which may bring into question the role of TNF-α in inducing abortion in *C. abortus*-infected mice.

Due to the unsuccessful T cell immunohistochemistry, the distribution of CD4$^+$ T cells in infected mouse tissues could not be determined. Resolution of this problem would have allowed identification of T cell populations with sites of infection and

cytokine expression. However, despite the lack of information on the T cell distribution, it can be concluded a Th1 type immune response was present in the *C. abortus* infected mice. The predominance of IgG2a antibodies and detection of TNF-α and IFN-γ $mRNA^+$ cells are all indicative of a strong Th1 immune response (Birkelund *et al.*, 2004; Igietseme *et al.*, 1998; Snapper & Paul, 1987), and suggest that the infection induced the putative protective immune response in the mouse model. However although the afore-mentioned cells and responses are characteristic of protective Th1 immunity, some of the factors may actually play a role in abortion. Infiltration of inflammatory cells and release of cytokines by these cells results in the recruitment of other cell types to the infection sites within the placenta, and this may cause severe pathological damage as well as controlling the infection. This occurs in ovine infections, where substantial levels of necrosis, probably as a result of the influx of inflammatory and other cells, in the interplacental areas and cotyledons are reported (Buxton *et al.*, 2002). Infiltration of infected cells during pregnancy is likely to compromise placenta function, and therefore interfere with foetal development, thereby resulting in abortion. Pregnancy failure in a mouse model has been linked to presence of the proinflammatory cytokine TNF-α, which was associated with impaired synthesis of progesterone by the corpus luteum and the induction of the prolactin receptor signaling suppressors of cytokine signaling 1 (SOCS1) and SOC3 (Erlbacher *et al.*, 2004). Progesterone, the hormone of pregnancy, decreases in infected sheep placenta and this may contribute to abortion and as previously mentioned, TNF-α levels increase during *C. abortus* infection of mice and sheep (Buxton *et al.*, 2002), further supporting its possible role in abortion. Immune activation and associated reproductive endocrine dysfunction are also likely to play a role in the induction of abortion in infected animals.

This study of immune responses to *C. abortus* in the pregnant mouse model has revealed similarities to the immune responses identified in the ovine host. An influx of inflammatory cells to sites of infection was observed in mice, which is similar to ovine infections, and a Th1 response appears to be dominant in both species, characterised by the expression of IFN-γ and TNF-α at both molecular and protein levels. Despite the obvious differences between mouse and sheep in placentation, the

similarities between *C. abortus* infection in the two species, in terms of abortion and immune responses, provides further evidence of the suitability of this pregnant mouse model for the initial assessment of potential vaccine candidates against *C. abortus*.

CHAPTER 5

INVESTIGATION INTO DEVELOPMENT OF LATENT *C. ABORTUS* INFECTIONS IN MICE

5.1 Hypothesis

Infection of non-pregnant mice with *C. abortus* will lead to the development of a latent infection and pregnancy will cause the organisms to recrudesce, resulting in abortion.

5.2 Introduction

The stage of pregnancy at which pregnant ewes are infected with *C. abortus* determines the outcome of infection. As previously mentioned, ewes that are infected during early pregnancy abort in the final trimester (Buxton *et al.*, 1990). In contrast, infection of non-pregnant sheep leads to development of a latent infection and the localisation of the organism during this phase remains unknown (Huang *et al.*, 1990; McEwen *et al.*, 1951; Wilsmore *et al.*, 1990). If sheep are infected late in pregnancy they similarly develop this latent infection (McEwen *et al.*, 1951). No matter when infection occurs, *C. abortus* organisms cannot be detected until the ewes become pregnant, and is first detected on day 90 of pregnancy when the infection recrudesces to cause abortion (Buxton *et al.*, 1990).

The location of *C. abortus* during the latent phase prior to recrudescence, has not been determined, though it is thought to be in lymphoid organs, with multiplication under the control of IFN-γ (Brown & Entrican, 1996). Hormonal shifts and changes in immune responses as a result of pregnancy are thought to alter the controlled latent state of the organisms, allowing them to become reactivated and infect the placenta, although the precise mechanisms remain unidentified (**Section 1.7**).

Despite the extensive use of mouse models to study immune responses to *Chlamydia* and to assess efficacy of vaccine candidates, no comparisons of the model with aspects of the ovine disease have been carried out to validate the mouse as a model for EAE. This experiment therefore investigated the development of latent infections and the recrudescence of organisms to cause abortion in mice for comparison to ovine infections.

5.3 Aim

To determine whether latent infections develop in non-pregnant mice by evaluating the incidence of abortion in the subsequent pregnancy, as occurs in the ovine host.

5.4 Materials and methods

5.4.1 Infection and mating of mice

Non-pregnant CBA ($H\text{-}2^K$) mice were infected by i.p. injection on day 0 with 10^6 IFUs of S26/3. Uninfected (negative control) animals were administered with 0.2 ml PBS via i.p. injection.

5.4.2 Initial investigation into latency in mice – experiment A

Twenty non-pregnant female mice were infected with S26/3 and ten non-pregnant female mice were inoculated with PBS, as described in **Section 5.4.1**. Two uninfected and four infected non-pregnant mice were euthanased on days 3 and 5 p.i. to confirm that mice were infected pre-mating (**Table 5.1**). The remaining mice were mated on day 7 p.i., (synchronised using the Whitten Effect, as described in **Section 4.4.1**) with males overnight (1 female: 1 male) and the presence of a vaginal plug denoted as day 0 of pregnancy. Two uninfected and four infected pregnant/mated mice were euthanased on days 22 and 24 p.i. and at the end of pregnancy (day 30 p.i.), i.e. post-littering or abortion depending on the outcome of disease, as shown in **Table 5.1**.

Table 5.1 Experiment A – plan of inoculations and serial kills in the initial investigation into latent *C. abortus* infections in mice

Group	n	Inoculum/pregnancy status	PM day p.i.	No. of mice killed/day
1 Uninfected	10	PBS day 0; mated day 7 p.i.	3, 5, 22, 24, 30	2
2 Infected	20	*C. abortus* day 0; mated day 7 p.i.	3, 5, 22, 24, 30	4

From each mouse, liver and uterus/foetal-placental units were collected into CTM to assess the recovery of live *C. abortus* organisms. Liver, spleen and foetal-placental units were also collected into 10% formal saline for histology and chlamydial detection studies.

The pregnancy rate was exceptionally low in this study, at approximately 18%, despite 100% vaginal plugs being present on day 0. It was decided that information on the recrudescence of latent infections was imperative to validate the mouse model of chlamydial abortion. Hence the experiment was repeated with a larger sample size in order to achieve a higher pregnancy rate so that the final outcome of pregnancy could be evaluated (Experiment B, **Section 5.4.3**).

5.4.3 Repeated study into the development of latency in mice - experiment B

Eighty-three non-pregnant female mice were infected with either S26/3 or PBS on day 0 (**Table 5.2**). In this study, 5 (3 from group 1 and 2 from group 2) non-pregnant infected mice were killed on each of days 3, 7 and 14 p.i. to verify that mice were successfully infected (day 3 p.i.) and to determine if *C. abortus* organisms had been cleared from the mice (days 7 and 14 p.i.). Two non-pregnant uninfected mice (group 3) were also euthanased on day 28 post-inoculation to obtain negative control tissues.

As shown in **Table 5.2**, 42 infected mice and 4 non-infected mice from groups 2 and 3, respectively, were mated on day 21 p.i.. Mice had been synchronised with the

Whitten Effect and were mated at a ratio of 1 female: 1 male. Group 1 mice were non-pregnant infected controls. Five infected non-pregnant mice (group 1), and 6 infected pregnant mice (group 2) were euthanased on days 28, 35 and 38 p.i. to establish any differences in infection between pregnant and non-pregnant mice. Two uninfected pregnant mice (group 3) were euthanased on day 38 p.i. to obtain chlamydial-negative liver, spleen, foetuses and placentae. Autopsies were carried out on the remaining 5 infected non-pregnant mice (group 2) on day 49 p.i.. Both infected (24 mice; group 2) and uninfected (2 mice; group 3) pregnant mice were allowed to abort or litter and *post-mortems* were conducted on days 49 and 50 p.i. Pups from infected animals were euthanased 7 days post-littering to determine the rate of survival during this period.

Table 5.2 Experiment B - plan of inoculations and serial kills in the repeated investigation into latent *C. abortus* infections in mice

Group	n	Inoculum/pregnancy status	Day p.i. of necropsies	No. of mice killed/day
1	29	*C. abortus,* non-pregnant mice	3, 7, 14 28, 35, 38, 49	3 5
2	48	*C. abortus* pre-mating, mated day 21 p.i.	3, 7, 14 28, 35, 38 49	2 6 24
3	6	Uninfected group, 4 mated day 21 p.i.	28 (non-pregnant) 38 49	2 2 2

Liver, spleen, mesenteric and hepatic lymph nodes and uteri were collected from non-pregnant animals for PCR detection of *C. abortus.* Liver, spleen and uterus samples were stored in CTM to be assessed for the culture of infectious organisms. From pregnant animals, the above tissues were removed as well as foetuses/pups depending on the stage of pregnancy. All samples were stored at -20°C until required.

5.5 Results

5.5.1 Experiment A - initial investigation into latency in mice

5.5.1.1 Outcome of experiment A

Since no infected pregnant mice were present at the end of the experiment, it was not possible to conclude whether mice had developed a latent infection by virtue of abortion. As previously mentioned, the pregnancy rate was only 18%, which was considerably lower than in previous studies.

5.5.1.2 Recovery of chlamydial organisms

Tissue samples were collected into CTM to assess the recovery of live *C. abortus* organisms to monitor infection levels in infected mice, as described in **Section 2.7**. **Figure 5.1** shows the number of organisms cultured on cell monolayers from tissue samples collected over the course of infection.

Recoveries of *C. abortus* from pregnant and non-pregnant mice were analysed separately and results are presented for individual animals to demonstrate the wide variation in titres in each group. **Figure 5.1** shows the successful infection of mice pre-mating, with liver titres of 2500-30000 IFUs/g. In non-pregnant mice (that were mated but unsuccessfully impregnated), liver tissue from one mouse was infected on day 22 p.i., and *C. abortus* organisms were cultured from uteri sampled on days 24 and 30 p.i. *C. abortus* was not identified in both uterus and liver of any mouse. Of the two infected pregnant mice killed on day 22 p.i., organisms were cultured from the uterus of one and the placenta of the other.

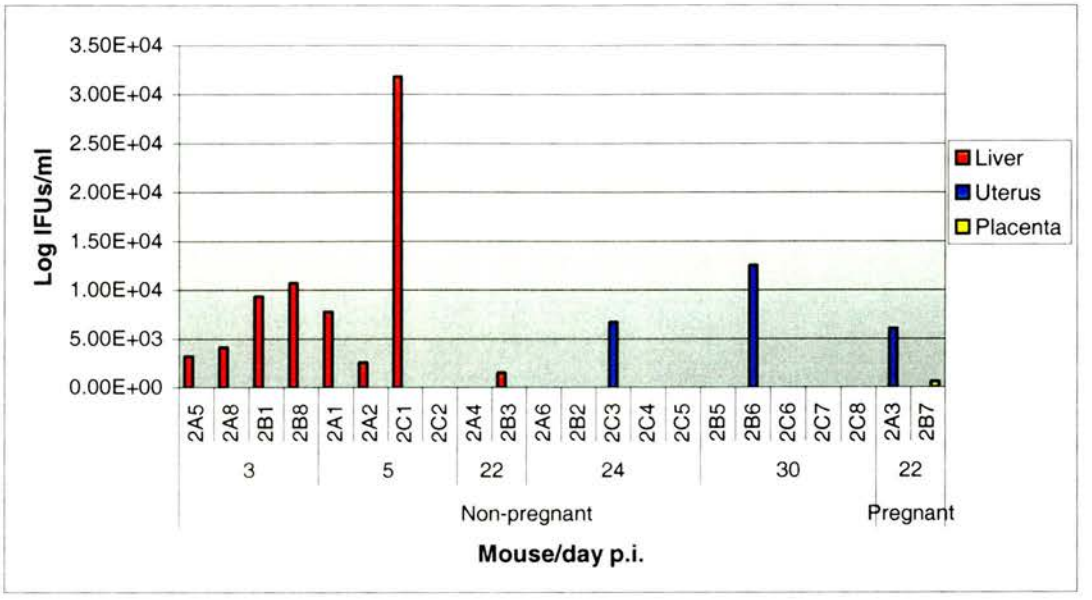


Figure 5.1 *C. abortus* recovery from infected pregnant and non-pregnant mice. Numbers of inclusions (log IFUs/g tissue) cultured from liver, uterus and placenta tissue from each mouse were counted under light microscopy and the IFUs/g calculated. Mice were mated on day 7 p.i. All uninfected mice were negative for *C. abortus* organisms.

5.5.1.3 Histological analysis

Histological analysis of sections stained with H & E revealed typical characteristics of *C. abortus*-infection in mice on days 3 and 5 p.i, with inflammatory infiltration present in liver tissues. By day 22 p.i., liver, uterine and placental tissues generally appeared uninfected although occasional small clusters of inflammatory foci were observed. All uninfected mice appeared normal.

5.5.2 Experiment B - repeated study into the development of latency in mice

5.5.2.1 Outcome of infection in experiment B

The pregnancy rate for mice mated in experiment B was 32%, almost double that of experiment A, although this was still lower than had been achieved previously (**Chapters 3** and **4**). Abortions did not occur in mice that were infected pre-mating and live, healthy pups were delivered as normal at term.

5.5.2.2 Culture of chlamydial organisms

To establish levels of infection in mice, live organisms were cultured (**Section 2.7**) from liver, spleen and uteroplacental units, where possible. Recovery of live *C. abortus* organisms was assessed for all 83 liver and spleen tissues samples (**Figure 5.2**), as well as for pregnant uteri and foetuses from pregnant mice and live pups at the end of the experiment. It is clear that mice were successfully infected (**Figure 5.2**), indicated by the culture of live organisms on day 3 p.i in liver and spleen tissue. Fewer inclusions were cultured on day 7 p.i. than on day 3 p.i. and this was in only 3 of 5 mice. No inclusions were detected in either liver or spleen on day 14 p.i..

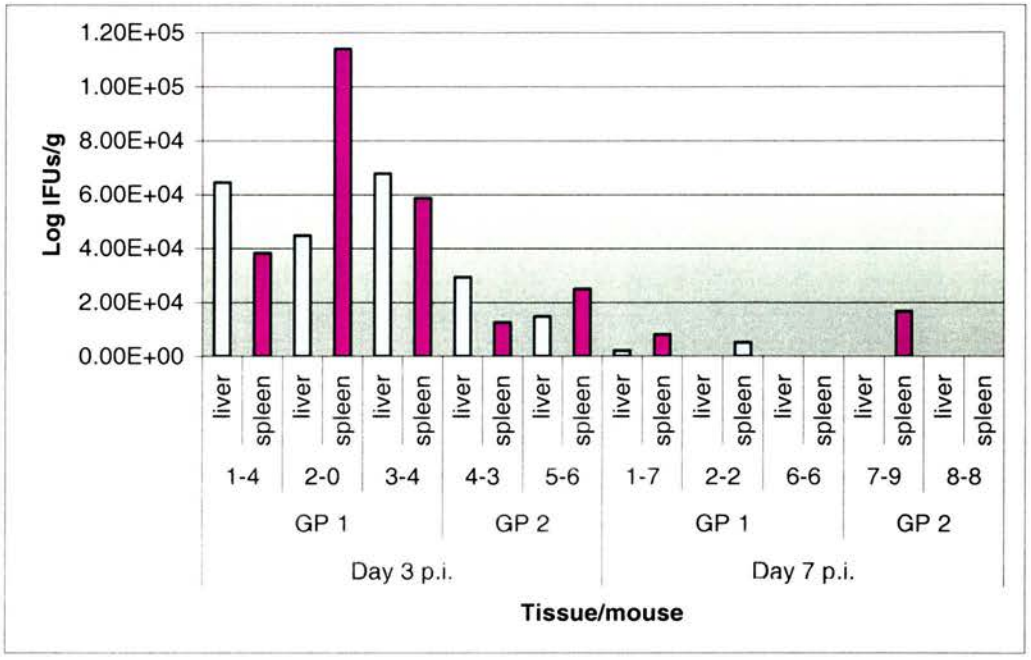


Figure 5.2 Recovery of organisms from infected mice. Log IFUs/g of tissue are presented for individual animals on days 3 and 7 p.i. for infected, non-pregnant mice. No inclusions were cultured from uninfected tissues or from infected non-pregnant liver or spleen tissues on days 14, 28, 35, 38, or 49 p.i. Mice were mated on day 21 p.i. Similarly no organisms could be recovered from liver or spleen samples or from foetuses or placental tissues collected from pregnant mice on days 28, 35, 38, 49 or 50 p.i..

5.5.3 Diagnostic PCR

5.5.3.1 Optimisation of diagnostic PCR

PCR is a highly sensitive tool for diagnosing *Chlamydia* infections, and could therefore increase the possibility of detecting low numbers of organisms compared with the previously used culture methods (**Sections 5.5.1.2** and **5.5.2.2**).

The PCR was optimised and sensitivity levels determined in both liver and spleen tissues (**Section 2.16.1**). PCRs were carried out on the following templates: **1**. Dilutions of EBs only (**Figure 5.3a**); **2**. EB-only eluate (eluate from dilutions of EBs processed by the Dneasy kit) (**Figure 5.3b**); **3**. Eluate from liver or spleen tissues spiked with dilutions of EBs then processed by the Dneasy kit (first 7 lanes in **Figure 5.3c,d**); and **4**. Eluate from liver or spleen tissues processed by the Dneasy kit, then spiked with dilutions of EBs (second 7 lanes in **Figure 5.3c,d**). Sensitivity of the PCR and loss of sensitivity due to sample processing through the Dneasy kit are summarised in **Table 5.3**.

Highest sensitivity was detected when PCR was conducted on dilutions of EBs that had been neither processed by the Dneasy kit nor mixed with tissue samples (**Table 5.3**). A loss in PCR sensitivity was apparent when reactions were conducted on EB dilutions alone compared with PCRs on tissues processed by the Dneasy kit and subsequently spiked with EBs. The spiking of EBs prior to tissue processing decreased the sensitivity of detection in liver tissue, however, increased the sensitivity of detection in spleen tissue.

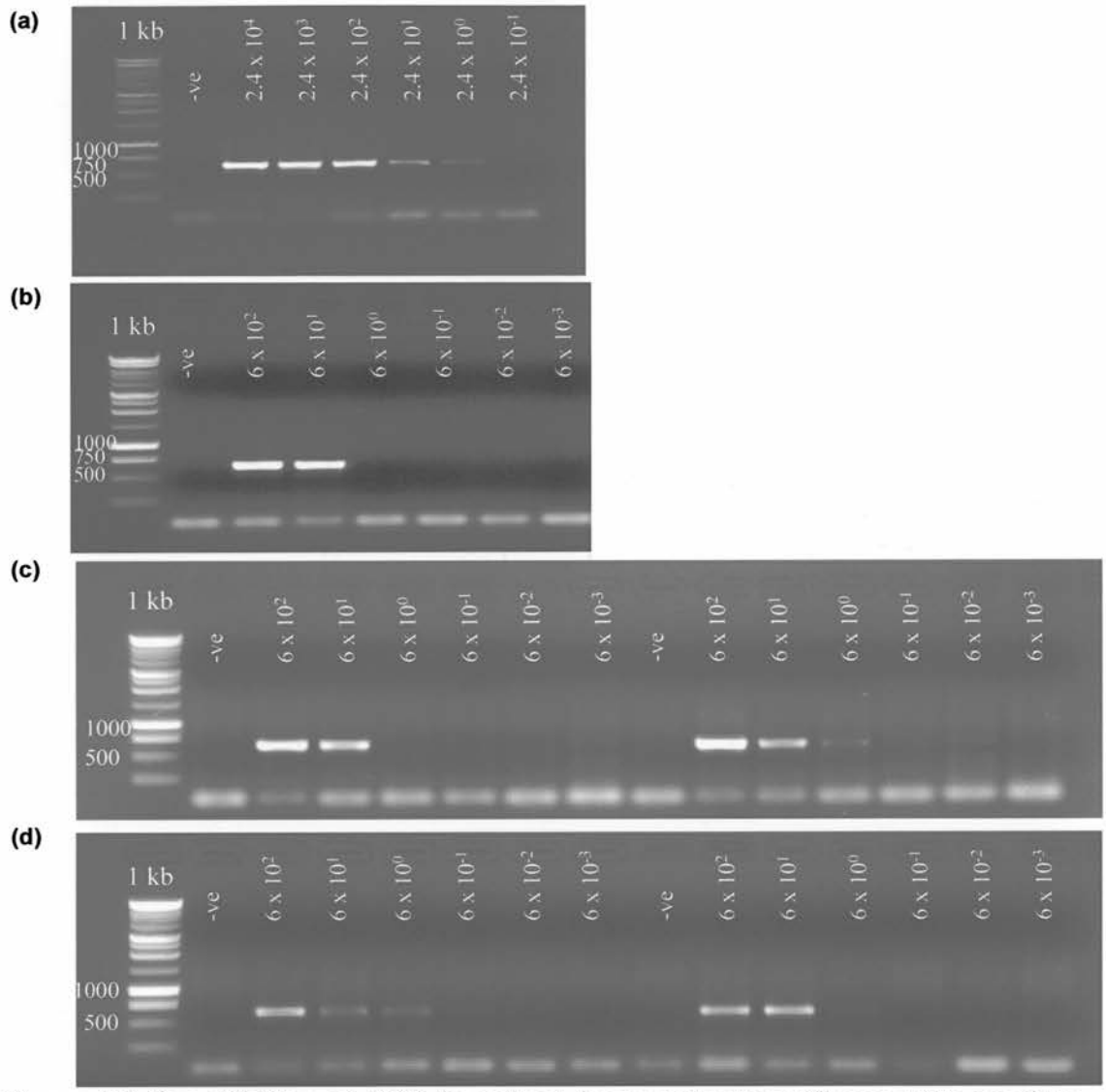


Figure 5.3 Sensitivities of PCR in spiked liver and spleen tissue with positive results shown at 640 bp on an agarose gel. (a) on EBs alone (control); (b) on EBs processed through kit; (c) on liver and (d) spleen tissues: the first 7 lanes represent tissue plus EBs processed through the kit, and second 7 represent tissue processed through the kit and EBs were then added to eluate. Numbers of IFUs are labelled.

Table 5.3 Sensitivity levels (IFUs) of PCRs using dilutions of EBs, and spiked liver and spleen tissues

Sample	EBs processed	EBs non-processed	Loss of sensitivity
EBs alone	60	2.4	25 X
Liver	60	6	10 X
Spleen	6	60	0

5.5.3.2 Sample assessment

In order to establish which mice were positive for *C. abortus* by PCR, 45 representative mouse liver and spleen samples from the different groups were processed through the Dneasy Kit, and the 16SF PCR was carried out (**Section 2.16.1.1**). Results from a range of samples that were assessed for the presence of *C. abortus* by PCR and were analysed on an agarose gel are shown in **Figure 5.4**.

Positive results were detected by PCR up to and inclusive of day 14 p.i. All samples were negative after this time point (**Table 5.4**) with no positive results detected in infected mice following mating on day 21 p.i. PCR appears to be a more sensitive tool for diagnosis as positive results were identified in mice that were identified as negative by culture (summarised in **Table 5.5**). PCR results were more sensitive and consistently positive on days 3, 7 and 14 p.i. although one negative result was recorded on day 7 p.i.

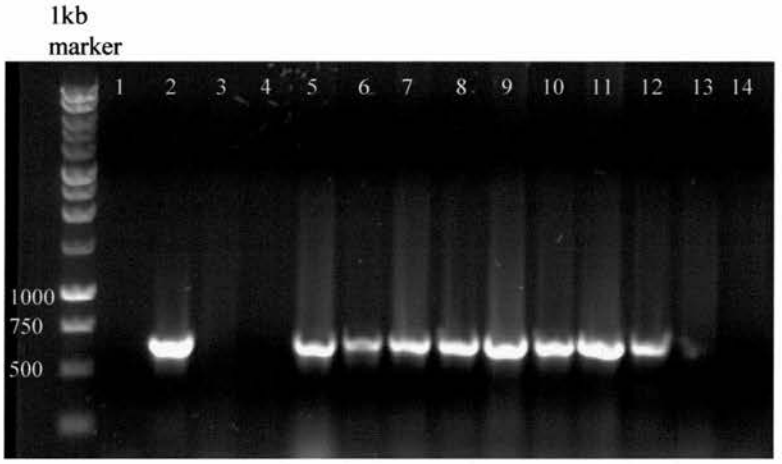


Figure 5.4 Representative PCR results in uninfected and infected liver and spleen tissues. PCR reactions were run on a 1% agarose gel, with a 1 kb marker, a negative control, dH_2O (lane **1**) and a positive control (25 ng genomic DNA) (lane **2**). Other samples were as follows: **3**-uninfected liver (day 28 p.i.), **4**-uninfected spleen (day 28 p.i.), **5**-infected liver (day 3 p.i.), **6**-infected spleen (day 3 p.i.), **7**-infected liver (day 7 p.i.), **8**-infected spleen (day 7 p.i.), **9**-infected liver (day 14 p.i.), **10**-infected spleen (day 14 p.i.), **11**-infected liver (day 14 p.i.), **12**-infected spleen (day 14 p.i.), **13**-infected liver (day 35 p.i.), **14**-infected spleen (day 35 p.i.).

Table 5.4 Summary of PCR results

	Group 1 Infected mice (non-pregnant)			Group 2 Infected mice mated on day 21 p.i.			Group 3 PBS controls mated on day 21 p.i.		
Day p.i.	**Mouse**	**Liver**	**Spleen**	**Mouse**	**Liver**	**Spleen**	**Mouse**	**Liver**	**Spleen**
3	1-4	+ve	+ve	4-3	+ve	+ve			
	2-0	+ve	+ve	5-6	+ve	+ve			
	3-4	+ve	+ve						
7	1-7	+ve	+ve	7-9	+ve	+ve			
	2-2	+ve	+ve	8-8	+ve	**-ve**			
	6-6	+ve	+ve						
14	3-5	+ve	+ve	6-5	+ve	+ve			
	4-8	+ve	+ve						
	5-2	+ve	+ve						
28	1-8	-ve	-ve	3-1	-ve	-ve	9-2*	-ve	-ve
	1-3	-ve	-ve	5-7	-ve	-ve	9-6*	-ve	-ve
	2-1	-ve	-ve	5-0*	-ve	-ve			
35	3-3	-ve	-ve	5-9	-ve	-ve			
	1-0	-ve	-ve	6-8	-ve	-ve			
				7-1	-ve	-ve			
				7-2	-ve	-ve			
				4-0*	-ve	-ve			
38	1-1	-ve	-ve	4-1*	-ve	-ve	9-9	-ve	-ve
				6-4*	-ve	-ve			
49	1-5	-ve	-ve	7-7	-ve	-ve			
	1-6	-ve	-ve	5-1*	-ve	-ve			
				5-5*	-ve	-ve			
				5-8*	-ve	-ve			
				6-3*	-ve	-ve			
50				4-2	-ve	-ve	9-0	-ve	-ve
				8-0	-ve	-ve			
				8-3	-ve	-ve			
				8-7*	-ve	-ve			

* Signifies pregnant animal

5.5.3.3 Culture and PCR as diagnostic techniques

Table 5.5 shows a comparison between the similarities of PCR amplification of chlamydial DNA and culture of *C. abortus* from infected liver and spleen tissues. Uninfected mice were negative by both PCR and culture.

Of 9 infected mice that were positive by PCR, only 4 were positive by culture (44%) from liver and 5 by culture (55%) from spleen. Overall, 12 liver samples were positive by PCR, with only 2 negative samples. In contrast only 6 samples were positive by culture. Similar results were obtained for spleen, with 12 positive and 2 negative results determined by PCR and 8 positive and 6 negative results by culture. PCR detected positive samples after day 7 p.i. while culture did not.

Table 5.5 PCR versus culture results in *C. abortus* infected mice

	Group 1					Group 2				
Day p.i.	**Mouse**	**Liver**		**Spleen**		**Mouse**	**Liver**		**Spleen**	
		PCR	Culture	PCR	Culture		PCR	Culture	PCR	Culture
3	1-4	+ve	+ve	+ve	+ve	4-3	+ve	+ve	+ve	+ve
	2-0	+ve	+ve	+ve	+ve	5-6	+ve	+ve	+ve	+ve
	3-4	+ve	+ve	+ve	+ve					
7	1-7	+ve	+ve	+ve	+ve	7-9	+ve	-ve	+ve	+ve
	2-2	+ve	-ve	+ve	+ve	8-8	+ve	-ve	-ve	-ve
	6-6	+ve	-ve	+ve	-ve					
14	3-5	+ve	-ve	+ve	-ve	6-5	+ve	-ve	+ve	-ve
	4-8	+ve	-ve	+ve	-ve					
	5-2	+ve	-ve	+ve	-ve					

5.6 Discussion

Non-pregnant mice were infected to establish whether a latent infection would develop and recrudesce to cause abortion once mice were pregnant, as is observed in latently infected non-pregnant sheep that are subsequently impregnated. The study was conducted twice as a result of a low pregnancy rate in the initial study. No organisms were detected at any point in pregnant mice and these did not abort in the second experiment. This suggests that the model is not identical to the ovine disease, as latent infections did not develop to cause abortion in mice. Sufficient organisms may not have been present to cause abortion. In addition, the protective immune response may have efficiently controlled the infection before it reached the placenta.

Pregnancy rates were significantly lower in both experiments A and B than those achieved in previous studies. Non-infected mice were mated, and subsequently infected at mid-pregnancy in **Chapters 3** and **4**, whereas in this experiment, mice were infected and subsequently mated. Only 7 days separated inoculation and mating in experiment A and this may have affected the fertility of the mice. However, no signs of infection were detected prior to this time point (day 5 p.i.) in liver, spleen or uterine tissues as revealed by detection of live organisms by culture. The time between infection and mating in the repeated (and amended) experiment B was increased three-fold to ensure the infection had cleared in the hope that this would not impair fertility. Despite this, the pregnancy rate remained relatively low, although it was higher than in experiment A. 'Footprints' and enlarged, reddened uteri in mated, but non-pregnant mice in experiment B were frequently observed. Footprints are raised areas of the uterus with an almost bulbous appearance, where there were previously foetuses. Some uteri were enlarged in terms of the overall diameter compared to non-pregnant uteri, which were characteristically very thin, and there also appeared to be an increased blood flow to the uterus, suggesting that foetuses may have been present but had been resorbed.

Generally, mice had cleared the infection between days 5 and 22 p.i. in experiment A. However, the recovery of *C. abortus* organisms from 3 non-pregnant mice implies

that mating or even early loss of pregnancy, hindered clearance of a few organisms at least. More importantly, organisms were recovered from the uterus and placenta from the two infected pregnant mice at day 22 p.i., suggesting either a slight recrudescence or the infection had not been effectively controlled. Although, infection levels were markedly lower than in tissues assessed on days 3 and 5 p.i., particularly in the placenta. Although it failed to establish whether infection pre-mating influences the outcome of pregnancy, this study provided further evidence that mice are extremely efficient in the clearance of organisms from tissues.

Inflammatory foci denominated by PMNs were observed on days 3 and 5 p.i. in liver tissue, corroborating with results obtained in **Chapter 4**, those of the mouse model developed by Buendia *et al.* (Buendia *et al.*, 1999), and sheep studies (Buxton *et al.*, 2002). Infiltration had dramatically decreased by day 22 p.i. and remained at a low level on days 24 and 30 p.i. This provides further evidence that PMNs are involved in preliminary control of *C. abortus* infection, and concurs with the studies of Buendia *et al.* (1999) in which the role of PMN was identified in primary but not secondary infections.

Abortion was not observed in experiment B and pregnant mice delivered live, healthy pups at term. *C. abortus* was not detected in either non-pregnant or pregnant mouse liver, spleen, uteri or placentae despite the increased time between infection and mating to allow a latent infection to develop, and the use of a more sensitive diagnostic tool. A persistent rather than a latent infection may have developed, defined as a long term association between *Chlamydia* and the host, in which the organism remains viable but in a culture-negative state (Rottenberg *et al.*, 2002). In terms of *C. abortus*, persistent infections are thought to occur post-abortion, as demonstrated by Papp and Shewen (Papp & Shewen, 1996), rather than in non-pregnant sheep where the infection is latent/dormant.

C. abortus was cultured from liver and spleen on day 3 p.i., but by day 7 p.i. mice were almost free from infection and by day 14 p.i., no organisms were detected using the culture method. PCR was more sensitive than culture and chlamydial DNA was

detected in all liver and spleen tissues collected on day 3 p.i. and in the majority of tissues collected on days 7 and 14 p.i.. This is in agreement with studies by Amin (Amin, 2003a) where PCR was more sensitive than culture for *C. abortus* diagnosis in tissue samples collected from aborted ewes. However, it is possible that the failure to culture Chlamydiae was due to the presence of intermediate forms that were non-infectious (Buxton *et al.*, 1996) and this is consistent with *in vitro* experiments in which persistent forms are detected following the addition of IFN-γ (Beatty *et al.*, 1994; Brown *et al.*, 2001; Entrican *et al.*, 2001; Rottenberg *et al.*, 2002). Following mating and sampling after day 14 p.i. in this mouse experiment, there were no signs of *C. abortus* recrudescence by culture, PCR or abortion. In contrast to mice, non-pregnant sheep show no signs of infection following initial infection, and as in routinely-infected (from contaminated tissues, aerosols etc.) pregnant sheep, there are no pathological symptoms until day 90 of pregnancy, with abortion as the first sign of infection (Buxton *et al.*, 1990). Several studies conducted (Amin, 2003a; Buxton *et al.*, 1996; Papp *et al.*, 1993) have shown that immunohistochemical detection of chlamydial antigen is more sensitive than culture. Buxton *et al.* (1996) showed PCR to the most sensitive method and suggested that it should the method of choice for detection of sites of latent infections.

Although no signs of infection were identified post-mating, it is possible that the infection recrudesced during pregnancy but the methods of diagnosis were not sensitive enough to detect it. PCR could detect *C. abortus* in tissues that were negative by culture, although the sensitivity was 60 IFUs and 6 EBs in liver and spleen, respectively, compared with 2.4 IFUs when the PCR was carried out on IFUs that had not been processed or added to tissues. Fortunately new PCR techniques are constantly being developed with higher specificity and sensitivities, and this may enhance diagnosis of infection in sheep. PCRs have been shown to have sensitivities from 0.25 IFUs down to 0.063 IFUs (Amin, 2003b; Madico *et al.*, 2000), with no differences between tissue culture inoculum and mouse liver spiked with inoculum (Amin, 2003b), in contrast to results in this study. These higher sensitivities were based on a touchdown enzyme time release-PCR rather than the conventional PCR used in this study. Only small pieces of tissues were processed for PCR analyses

(25 mg liver, 10 mg spleen), so one must bear in mind that there is a possibility if negative results are obtained, organisms could be in another area of the tissue. Ideally screening multiple areas of an organ would provide a more representative analysis, however there disadvantages of time and expense associated with this.

It is also possible that *C. abortus* organisms could have been residing in lymph nodes as is suggested to occur in ovine infections (Papp *et al.*, 1994). It would therefore be useful to assess the lymph nodes in mice for the presence of *C. abortus* by both culture and PCR methods in future studies. A systemic infection was established in the mice and the reticuloendothelial organs infected by this route were assessed, providing appropriate information on the infection, or lack of, in pregnant mice. A latent or persistent state of infection results from deprivation of host tryptophan, for which *C. abortus* is auxotrophic for its growth and multiplication (Entrican, 2002). IFN-γ is released in response to *C. abortus* infections and the cytokine induces expression of IDO, which in turn degrades the host tryptophan to kynurenine. In human and mouse placentae, tryptophan is degraded through constitutive expression of IDO in trophoblast cells (Entrican, 2002). Currently, there is no information of the expression of IDO in the ruminant placenta. *C. abortus* lacks the *trp* operon and therefore cannot synthesise its own tryptophan, yet the organism requires it for growth and can succeed in growing in the placenta. This is probably possible due to temporal differences in IDO expression and the intricate immunological and physiological interactions during pregnancy, although exact details are unknown (Entrican *et al.*, 2004). Thus IFN-γ does not necessarily programme the host cell to kill the organisms as first thought and can be involved in the maintenance of persistence in the host (Entrican *et al.*, 2004).

These two studies conducted on latent infections in non-pregnant mice illustrate that the mouse is not a perfect model for the ovine disease in terms of similarity with all aspects of the disease. This is also the situation with other *Chlamydia* infections such as the *C. trachomatis* (MoPn) mouse model for human infections, whereby mice develop immunity to reinfection unlike humans, which can be repeatedly reinfected (Morrison & Caldwell, 2002). However, one must remember that rodent models are

used widely for the reasons of cost, time and ability to manipulate the immune responses and the greater availability of reagents for mice than larger mammals. Organisms are undetectable in non-pregnant sheep, and are believed to remain in a latent or dormant state until favourable conditions in pregnancy lead to reactivation and multiplication of *C. abortus* in the placenta. Even if mice in these experiments did not clear the infection or a latent infection developed, the onset of pregnancy did not trigger the reactivation of the infection. This may be attributed to differences in placental structures of mice and sheep.

To date, there are no reports in the literature of experimental models of latency in sheep. Information on the exact location of *C. abortus* organisms during subclinical persistent infections would prove exceptionally useful in terms of diagnosis and control. Close examination of changes in immune responses and hormonal changes would also reveal vital information regarding control and reasons for recrudescence. Research into latent infections of *C. abortus* and persistent infections of other *Chlamydia* species is continually progressing and will help in elucidating the reasons behind persistence, revealing new possible targets of control. Despite its differences regarding latency, this mouse model of chlamydial abortion will be of great benefit in terms of screening vaccine candidates that can then be assessed in the natural host, should they appear to offer an indication of protection to abortion following *C. abortus* infection.

CHAPTER 6

ANALYSIS OF IMMUNITY TO ABORTION

6.1 Hypothesis

Re-challenging post-abortion results in a reduction in the number of abortions, indicating immunity to disease, as occurs in the ovine host.

6.2 Introduction

The immune response to *C. abortus* infection has yet to be fully defined and studies to identify the prevalent immune responses are ongoing. *C. abortus*-infected ewes that abort in one pregnancy are immune to abortion in the subsequent pregnancy and this immunity to abortion has also been demonstrated to experimental secondary infection (Papp *et al.*, 1994). Further investigation of this 'natural' immunity to abortion will provide insight into mechanisms of the protective immune response. That ewes seroconvert following abortion has been previously demonstrated (Aitken, 2000), however, as with the other Chlamydiales, the cell mediated immune response against *C. abortus* is more important than the humoral response in terms of control (Guagliardi *et al.*, 1989; Igietseme *et al.*, 2002).

Some sheep develop sterile immunity, other animals continue to shed *C. abortus* organisms for up to 3 years post-abortion (Papp *et al.*, 1994), however the differences in immunity/sterility between the animals is not understood, knowledge of which is essential for vaccine development. Organisms were identified in placental tissue and post-partum fluids of immune sheep (that had previously aborted) in the subsequent pregnancy post-abortion yet abortion did not occur in these sheep, although infection levels were significantly lower than in primary-infected sheep (Papp *et al.*, 1994; Papp & Shewen, 1997).

Experiments in the mouse model have examined immune responses to *C. abortus*, showing similarities to those responses observed in infected ewes (Buendia *et al.*, 1999; Del Rio *et al.*, 2000) and vaccines have been tested in pregnant mouse models (Caro *et al.*, 2003; Hechard *et al.*, 2003a; Hechard *et al.*, 2003b) proving the usefulness of animal models. However, no studies have been conducted to determine

how suitable the pregnant mouse model is as a model for ovine chlamydial infections in terms of immunity post-abortion. Such studies will provide valuable information on immune responses and also show further similarities and/or differences between the model and the natural ovine host.

6.3 Aim

To further validate the mouse model by investigating immunity to abortion in the subsequent pregnancy post-abortion to progress understanding of immunity and to allow comparison with the ovine disease.

6.4 Materials and methods

6.4.1 Experimental plan – mating and infection of mice

CBA (H-2^K) mice were mated (synchronised using the Whitten Effect, **Section 4.4.1**), allowed to go to term, and mated again 3 weeks post-term (**Table 6.1**). Mice were inoculated by i.p. injection with 0.2 ml 10^6 IFUs of S26/3 or with PBS on day 11 of pregnancy, details shown in **Table 6.1**.

Mice were killed by CO_2 and cardiac puncture. Following removal of the uterus, foetuses were killed immediately by cervical dislocation. Samples of foetoplacental units were collected into CTM to examine recovery of infectious organisms (**Section 2.7**), and into various fixatives for immunopathological studies (**Section 2.8**). In situations where mice were not successfully impregnated, the uterus was sampled. Serum was collected from blood samples to identify *Chlamydia*-specific antibody isotypes (**Section 2.9**) and liver tissues were fixed for various immunopathological and *in situ* hybridisation studies (**Section 2.11.5**). Liver and placenta samples were snap-frozen for T cell immunodetection studies and liver tissue was also stored in CTM for culture of infectious organisms. Spleen tissue was collected into MWM for the following immunological assays: lymphocyte stimulation assays, flow cytometry

analysis and stimulation of splenocytes for cytokine analysis (**Sections 2.13**, **2.14** and **2.15**, respectively).

Table 6.1 Experimental plan of investigation into immunity to *C. abortus* infection in mice

Group		n	Pregnancy 1 (day 11)	Pregnancy 2 (day 11)	PMs post-inoculation in the 2nd pregnancy
1	Uninfected control	6	PBS	PBS	2 mice killed on the equivalent of days 3 and 5 p.i.; 2 mice killed post-littering
2	Experimental - primary infection	12	*C. abortus* (S26/3)	PBS	4 mice killed on days 3 and 5 p.i.; 4 mice killed post-abortion/littering
3	Experimental - secondary infection	12	*C. abortus* (S26/3)	*C. abortus* (S26/3)	4 mice killed on days 3 and 5 p.i.; 4 mice killed post-abortion/littering
4	Inoculum control in the second pregnancy	6	PBS	*C. abortus* (S26/3)	2 mice killed on days 3 and 5 p.i.; 2 mice killed post-abortion/littering

NB. Final PMs were carried out 1 week post-littering/abortion (day 22 p.i. secondary inoculation).

6.5 Results

6.5.1 Outcome of each pregnancy

68% of mice were successfully pregnant in the first pregnancy compared to only 43% in the second pregnancy. **Figure 6.1** shows the decrease in pregnancy rate observed in all groups following the second mating.

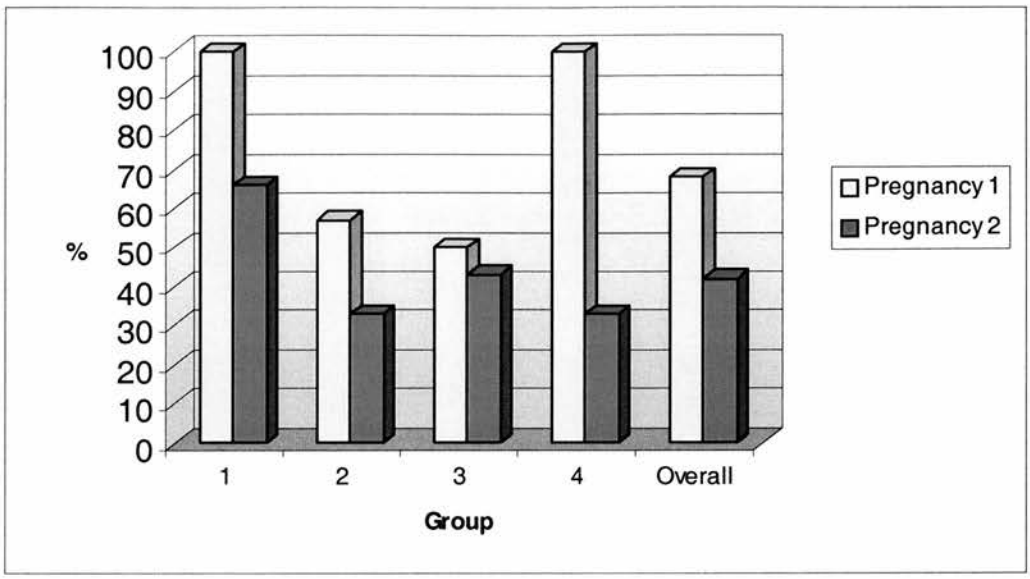


Figure 6.1 Pregnancy rates of uninfected and *C. abortus*-infected mice. The percentage of pregnant mice in each group of mice is presented, alongside the overall percentage for pregnancies 1 and 2.

Uninfected mice littered live, healthy pups in both pregnancies (**Table 6.2**). Infection of mice (groups 2 and 3) with *C. abortus* in the first pregnancy resulted in abortion on day 6-8 p.i. (days 17-19 of pregnancy), as expected. Group 2 mice did not abort in the second pregnancy after inoculation with PBS; live, healthy pups were littered and the offspring survived for at least 7 days. Secondary infection in the second pregnancy of group 3 mice also resulted in the littering of live, healthy pups, indicating immunity to abortion following re-infection. Mice littered following inoculation with PBS in the first pregnancy and aborted in the second pregnancy after infection with *C. abortus* (group 4), verifying the infectivity of the inoculum. The number of mice killed at each time point after the second inoculation is shown in **Table 6.3** and, unless otherwise stated, results throughout this chapter refer to these animals killed post-second inoculation in the second pregnancy.

Table 6.2 Pregnancy outcome in mice infected with *C. abortus* in two pregnancies

Group	Pregnancy 1	Pregnancy 2
1 – Uninfected mice [PBS in P1 and P2]	Littered	Littered
2 – Experimental group [S26/3 in P1, PBS in P2]	Aborted	Littered
3 – Experimental group [S26/3 in P1 and P2]	Aborted	Littered
4 – Inoculum control in P2 [PBS in P1]	Littered	Aborted

P=PREGNANCY.

Table 6.3 Number of uninfected and *C. abortus* infected mice killed at each time point

	Group 1			Group 2			Group 3			Group 4		
	3	**5**	**22**	**3**	**5**	**22**	**3**	**5**	**22**	**3**	**5**	**22**
Pregnant	2	1	1	1	1	3	2	1	3	1	0	1
Non-pregnant	0	1	1	3	4	1	3	4	1	1	2	1

Numbers 3, 5 and 22 represent the days p.i. that mice were killed post-secondary inoculation.

6.5.2 Culture of *C. abortus* organisms

Chlamydial inclusions were detected by culturing samples of homogenised tissues, collected from control and infected mice, on McCoy cell monolayers (**Section 2.7**). *C. abortus* was recovered from aborted placental samples from group 2 and 3 animals following infection in pregnancy one and from group 4 mice following infection in pregnancy two, varying from 6.98×10^2 to 4.77×10^5 IFUs/g of placenta. All samples from uninfected mice (group 1) were negative for *C. abortus* organisms.

Tissues from mice that were infected in the first pregnancy but were inoculated with PBS only (group 2) or EBs (group 3) in the second pregnancy were negative following secondary inoculation. Only samples collected from group 4 after the second pregnancy were positive for *C. abortus* organisms (**Table 6.4**).

Table 6.4 Recovery of *C. abortus* organisms from infected mice after the second pregnancy

	Mouse	Day of pregnancy	Day p.i.	Liver	Uterus	Placenta
Pregnant	4A1	14	3	$9.84x10^3$	n/a	$4.48x10^4$
	4A2	Post	22	0	0	$2.69x10^4$
Non-pregnant	4A3	14	3	$5.21x10^3$	0	n/a
	4A6	16	5	$1.44x10^4$	$1.09x10^4$	n/a

Numbers equate to IFUs/g of tissue. Placenta from 4A1 was collected from the uterus. 4A2 was killed on day 22 p.i., but the mouse aborted and the placenta was collected on day 7 p.i.

6.5.3 Histology and chlamydial antigen detection

Histopathological investigation (**Section 2.8.1**) revealed differences between groups of mice. All sections from uninfected mice (group 1) appeared normal with no necrosis or inflammatory cell infiltration. The majority of tissue sections sampled from group 2 (post-second pregnancy) had inflammatory foci present, although these were smaller in size and less frequent than those observed in tissues from the inoculum control (group 4) mice and some appeared similar to uninfected sections. Inflammatory foci were generally observed in tissues collected from secondary-infected mice (group 3), although foci were smaller in size compared to those observed in inoculum control mice (group 4). Slightly more inflammatory foci were observed in tissue sections of group 3 than those of group 2 mice. Necrosis was not observed in liver or placental tissues, agreeing with previous studies (**Chapters 3** and **4**). All uninfected mice (group 1) and mice from groups 2 and 3 were negative for

chlamydial antigen following the second pregnancy, as determined by immunohistochemistry (**Section 2.8.2**). Chlamydial antigen was detected only on days 3 and 5 p.i. in tissues sampled from group 4 mice, which were infected with *C. abortus* in the second pregnancy.

6.5.4 Identification of immune cell phenotypes

6.5.4.1 Detection of PMNs in liver

Focal inflammation was present in liver sections from all groups of infected mice (pregnant and non-pregnant mice) (**Figure 6.2**), with significantly higher numbers of PMNs in group 4, which had a primary infection of *C. abortus* in the second pregnancy, than within other groups ($F_{3,36}$=28.62, $p<0.001$). Despite group 3 mice being infected with *C. abortus* for a second time in the second pregnancy, a similar infiltration of PMNs was observed during the course of infection to that of group 2 mice, which were not given secondary infection in the second pregnancy. To assess similarities between pregnant and non-pregnant mice, PMN infiltration was compared, with similar numbers detected on day 3 p.i. in both groups of mice and significantly more cells present in liver tissues from all groups of pregnant mice on day 22 p.i. and in all groups of non-pregnant mice on day 5 p.i. ($F_{2,36}$=6.35, $p=0.007$).

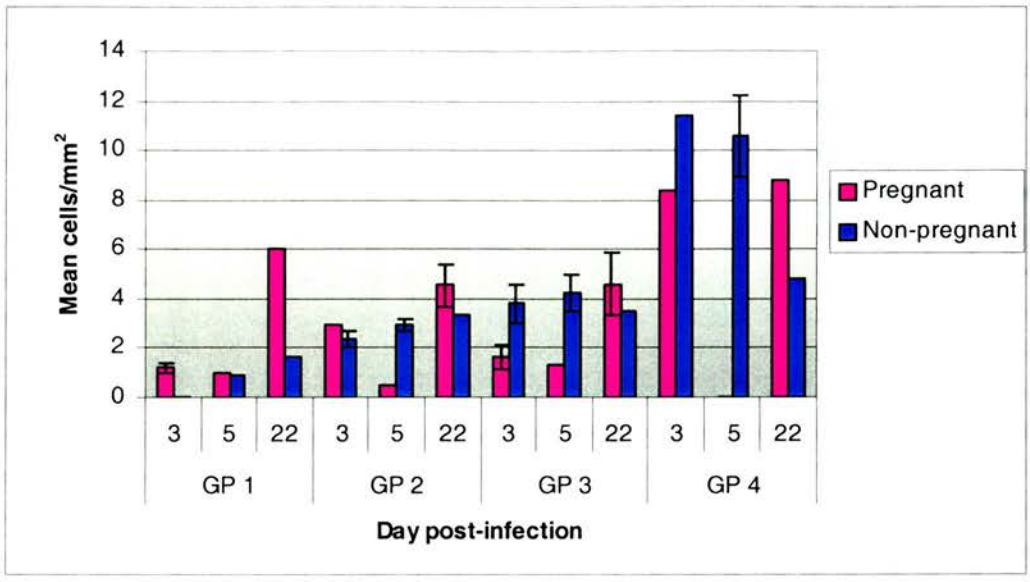


Figure 6.2 Ly-6G$^+$ cell detection in liver tissue from control and infected pregnant and non-pregnant mice after the second inoculation. Mean (± SEM) numbers of positive cells/mm^2 were calculated for pregnant and non-pregnant mice of each group on each day p.i. NB. No non-pregnant mice were present in group 1 on day 3 p.i. and no pregnant mice were present in group 4 on day 5 p.i. Where there are no error bars, there was only one mouse killed at that time point **(Table 6.3)**.

6.5.4.2 Detection of B cells in liver

Similar to PMN infiltration, higher numbers of B cells were detected in group 4 (primary infection in the second pregnancy) than within other groups of mice (pregnant and non-pregnant) ($F_{3,36}$=8.74, p<0.001) (**Figure 6.3**), although fewer B cells were present in all groups of mice than PMNs. Similar numbers of B cells were observed in sections of group 2 and 3 mice, which were immune to abortion in the second pregnancy. The presence of B cells in inflammatory foci were compared between pregnant and non-pregnant mice but this revealed no significant differences ($F_{1,36}$=0.35, p=0.562). No significant differences were detected at the different sample times of post-infection in either pregnant or non-pregnant mice ($F_{2,36}$=0.06, p=0.945).

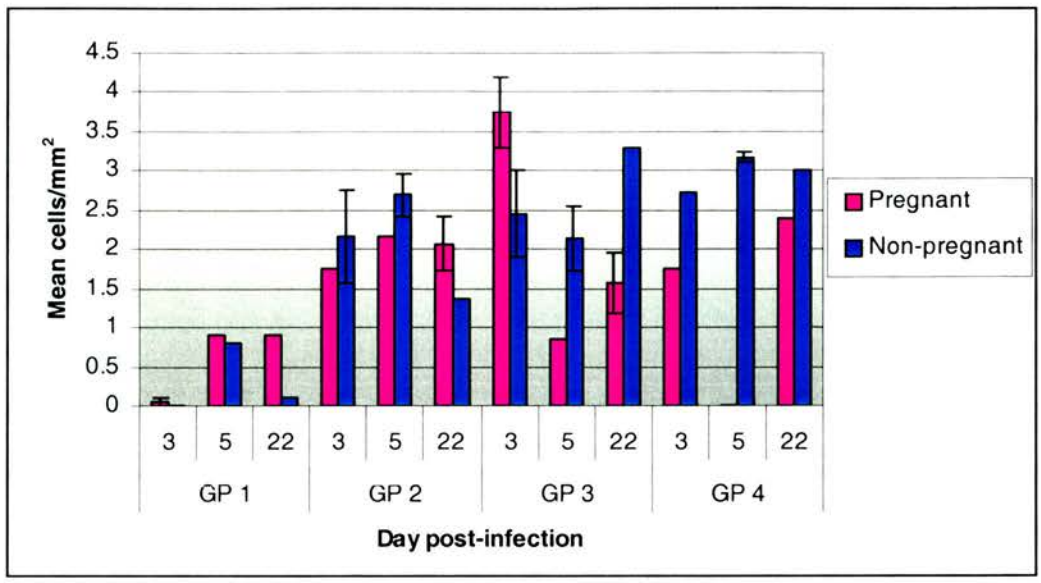


Figure 6.3 B220$^+$ cell detection in liver tissue from control and infected pregnant and non-pregnant mice after the second inoculation. Mean (± SEM) numbers of positive cells/mm^2 were calculated for pregnant and non-pregnant mice of each group on each day p.i. NB. No non-pregnant mice were present in group 1 on day 3 p.i. and no pregnant mice were present in group 4 on day 5 p.i. Where there are no error bars, there was only one mouse killed at this time point (**Table 6.3**).

6.5.4.3 Detection of PMN and B cells in placental tissue

Few placental samples from mice were collected into ZSF due to the low number of foetuses present in the uteri of some mice and because generally when mice litter, the mother eats the placental tissue. Lower numbers of PMNs were detected in the foetal labyrinth and chorionic plate areas than in maternal areas of the placenta in all of the placental sections that were analysed (**Figure 6.4**). Despite mice being immune (groups 2 and 3) and the lack of recovery of live organisms from placenta of these mice, an inflammatory response was present as was observed in liver tissues (**Figure 6.2**). Ly-6G$^+$ cells were detected in different areas of the placenta in samples from group 3 mice with highest numbers present in placental tissue from a group 3 mouse (3A2), which was killed on day 3 p.i.. This mouse had higher numbers of positive cells than those detected in placenta from the other mouse that was killed on day 3 p.i. (3A8) and the mouse killed on day 5 p.i. (3B5) in this group 3. B220$^+$ cells were restricted to the maternal areas of placental tissue in all mice, and overall the number

of B cells in each area was less than that of PMNs, agreeing with previous results shown in **Chapter 4**.

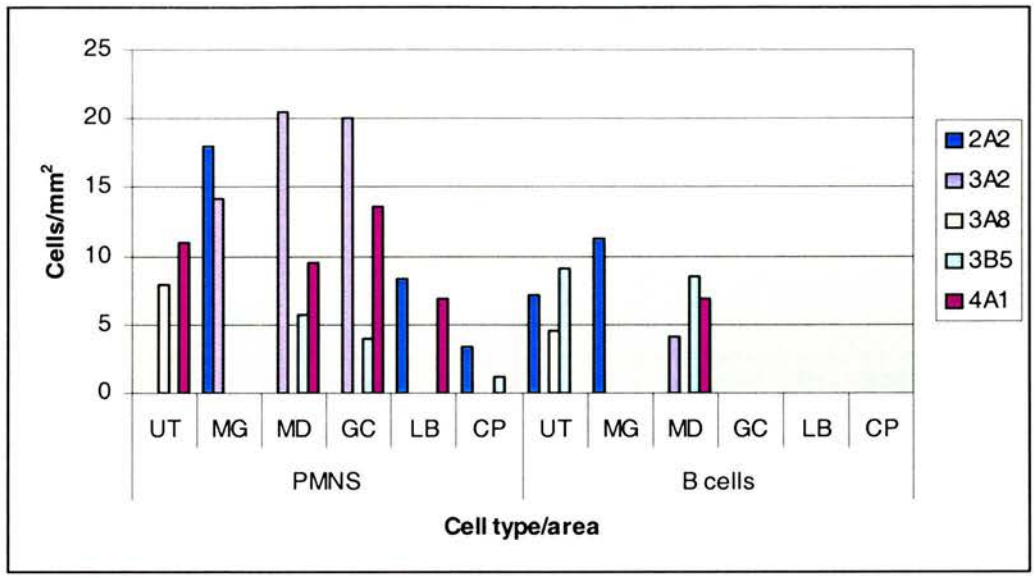


Figure 6.4 Ly-6G$^+$ and B220$^+$ cell detection in uteroplacental units in control and infected mice after the second inoculation. Numbers of positive cells/mm^2 were determined in different areas of uteroplacental units for individual mice. All mice were sampled on day 3 p.i. except 3B5 which was collected on day 5 p.i. Prefix 1, 2, 3 or 4 signifies the group (**Table 6.1**). UT=uterine epithelium, MG=metrial gland, MD=maternal decidua, GC=giant cells, LB=labyrinth, CP=chorionic plate.

6.5.4.4 T cell detection in liver and placental tissues

To determine the cellular response, T cells were detected by immunohistochemistry on representative samples of frozen tissue sections. This was carried out at the University of Murcia, as described in **Section 2.8.4.2**. CD4$^+$ and CD8$^+$ T cells were detected in all tissue sections that were analysed (**Figures 6.5** and **6.6**).

Immunophenotypical analysis of the lymphocyte infiltrate showed that CD4$^+$ T cells were the predominant population in the inflammatory foci of mice (**Figure 6.5**). A significantly greater number of CD4$^+$ than CD8$^+$ T cells were present in all of the liver sections that were analysed ($F_{1,28}$=8.23, p=0.009). Numbers of CD4$^+$ cells were comparable between secondary infected mice (group 3) on day 5 p.i. and inoculum

control (group 4) mice on days 5 and 22 p.i. in liver tissue. $CD4^+$ T cells were variable between mice within the same groups, whether pregnant or not, with the highest number of these cells present on day 5 p.i. in immune (group 3) mice. $CD4^+$ T cells were also numerous on day 3 p.i. (2A2) and on day 22 p.i. (4A2). In contrast, the number and distribution of $CD8^+$ T cells varied less between individual mice, although there were slightly more cells present in two mice from group 4 (**Figure 6.5**). No significant differences were determined between the number of $CD4^+$ or $CD8^+$ T cells between the groups of mice ($F_{2,28}$=1.00, p=0.413).

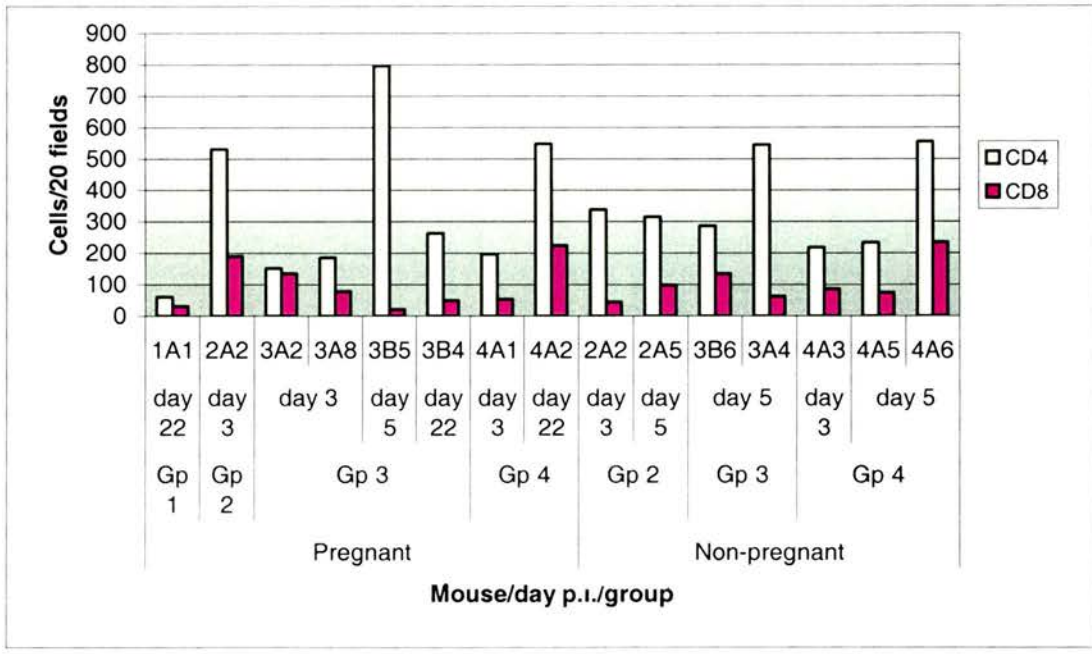


Figure 6.5 $CD4^+$ and $CD8^+$ T cell counts in liver tissues from individual mice after the second inoculation in the second pregnancy. Total numbers of positive T cells were counted in 20 fields of view by light microscopy (x20 magnification) on immunolabelled liver sections.

In all liver sections positive cells were scattered throughout the parenchyma, with $CD4^+$ T cells present in clusters and $CD8^+$ T cells generally present as individual cells (**Figure 6.6**).

(a) (b)

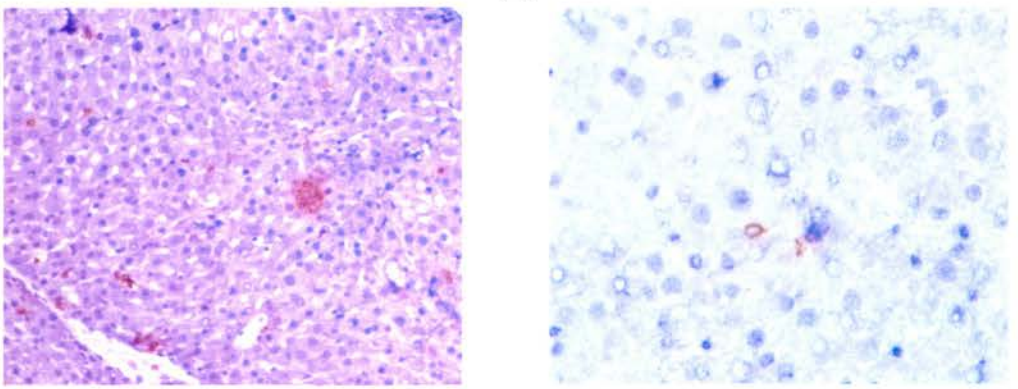


Figure 6.6 T cell staining in liver sections from pregnant mice infected with *C. abortus*. (a) $CD4^+$ and **(b)** $CD8^+$ T cells in liver tissue from an immune mouse (group 3) killed on day 5 p.i.

Four uteroplacental tissue samples were analysed for the presence of T cells at the University of Murcia. The majority of $CD4^+$ and $CD8^+$ T cells were expressed in the uterine epithelium, although T cells were also detected in the foetal labyrinth area (**Figure 6.7**). T cells were detected in uteroplacental tissues from immune (group 3) mice only but were not detected in the aborted placental sample (4A1) from the inoculum control group 4. As in liver sections, a higher number of $CD4^+$ than $CD8^+$ T cells were detected in placental sections (**Figure 6.7**). Images of representative positive sections are shown in **Figure 6.8**.

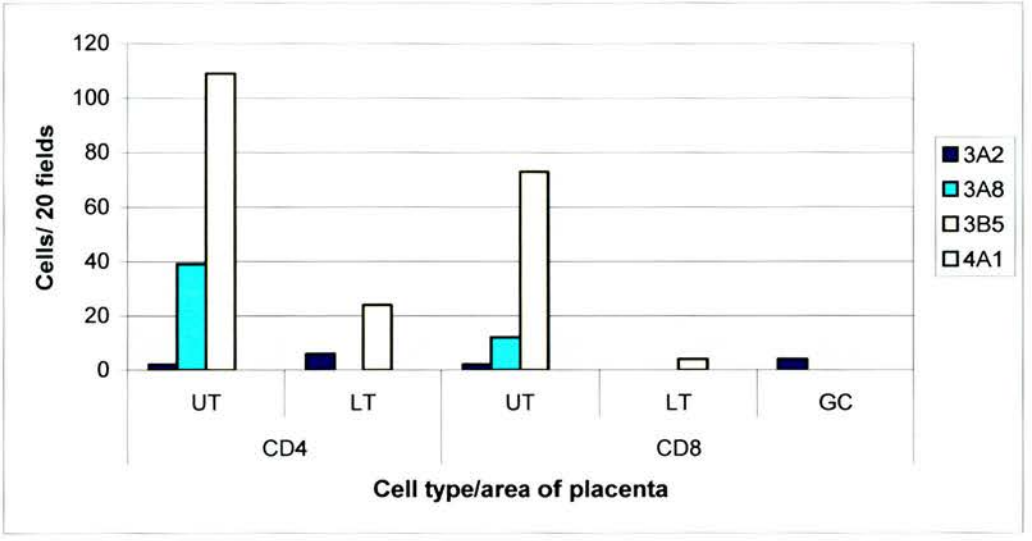


Figure 6.7 T cell distribution within infected placental tissue after the second inoculation. Total numbers of $CD4^+$ or $CD8^+$ T cells were counted in 20 fields of view by light microscopy (x20 magnification). UT=uterine epithelial cells, LT=labyrinth trophoblast cells, GC=giant cells. Prefix 2, 3 or 4 denotes group. Mice 3A2 and 3A8 were killed on day 3 p.i., 3B5 on day 5 p.i., and 4A1 represents an aborted placenta on day 7 p.i.

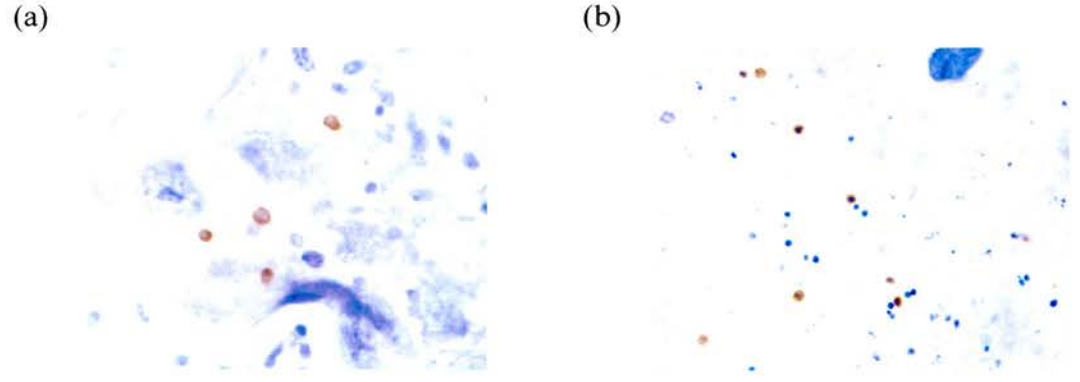


Figure 6.8 T cell staining in *C. abortus*-infected placenta sections. (a) $CD4^+$ cells at the edge of the trophoblast labyrinth area. **(b) $CD8^+$** cells within the uterine epithelium. Distribution of $CD8^+$ cells was very similar to $CD4^+$ T cell distribution in the uterus of infected mice.

6.5.4.5 NK cell detection in liver and placenta tissues

To determine if NK cells were expressed in response to *C. abortus* infection in mice, immunohistochemical labelling of frozen sections with an anti-Ly49G was conducted at the University of Murcia (**Section 2.8.4.2**). NK cell detection was successful in the positive control section kindly provided by Assistant Professor Antonio Buendia, however all of the sections from this study that were analysed were negative for NK cells.

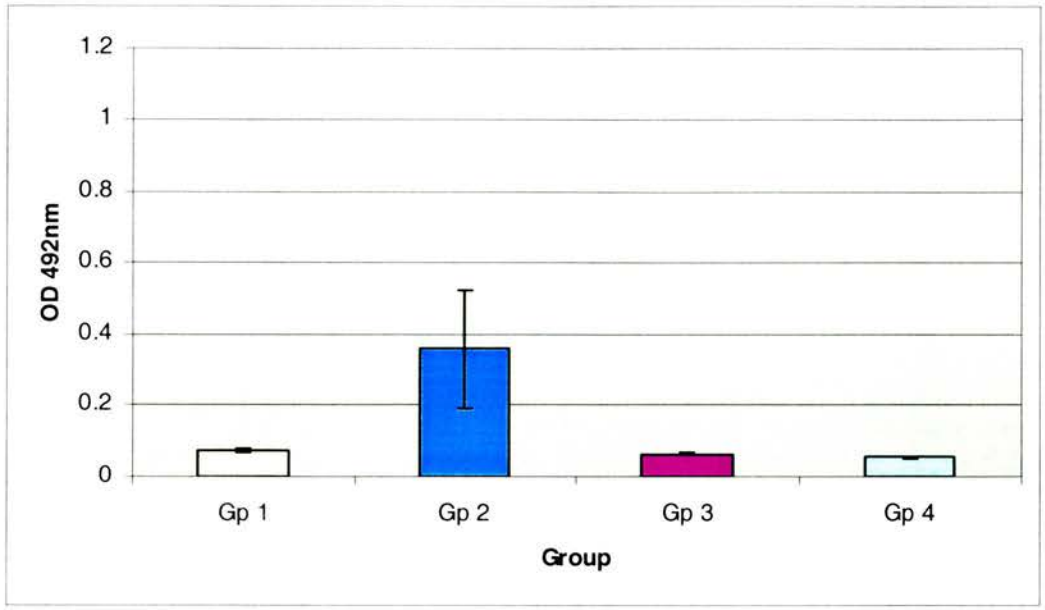
6.5.5 Antibody responses

6.5.5.1 ELISA results

C. abortus-specific antibody responses in mice were determined by analysis of sera by EB-specific ELISA, as described in **Section 2.9.1**. **Figures 6.9**, **6.10** and **6.11** show IgG1, IgG2a and IgM responses, respectively. Blood samples were collected at 3 different time points: **1.** Pre-infection by tail snips (before pregnancy 1; day 0) (data not shown as all results were negligible); **2.** Between pregnancies 1 and 2 by tail snips (equates to day 40 after the primary inoculation); and **3.** At the final PM by cardiac puncture on 3, 5 or 22 days post-secondary inoculation.

IgG1, which is indicative of a Th2 immune response, had significantly higher responses in group 2 ($F_{3,32}$=3.35, p=0.031) after the first pregnancy as shown in **Figure 6.9a**, despite mice in groups 2 and 3 being infected with exactly the same EBs. IgG1 levels remained high within group 2 following the second pregnancy, except on day 5 p.i. (**Figure 6.9b**). IgG1 responses were low in all mice after the second pregnancy, except those from group 2, which were significantly higher than in the other groups ($F_{3,36}$=4.14, p=0.016). Responses were similar at all other time points throughout infection/pregnancy ($F_{2,36}$=0.84, p=0.446) in all groups. Similar responses were also detected in pregnant and non-pregnant mice ($F_{1,36}$=0.01, p=0.944) following secondary inoculation.

(a)



(b)

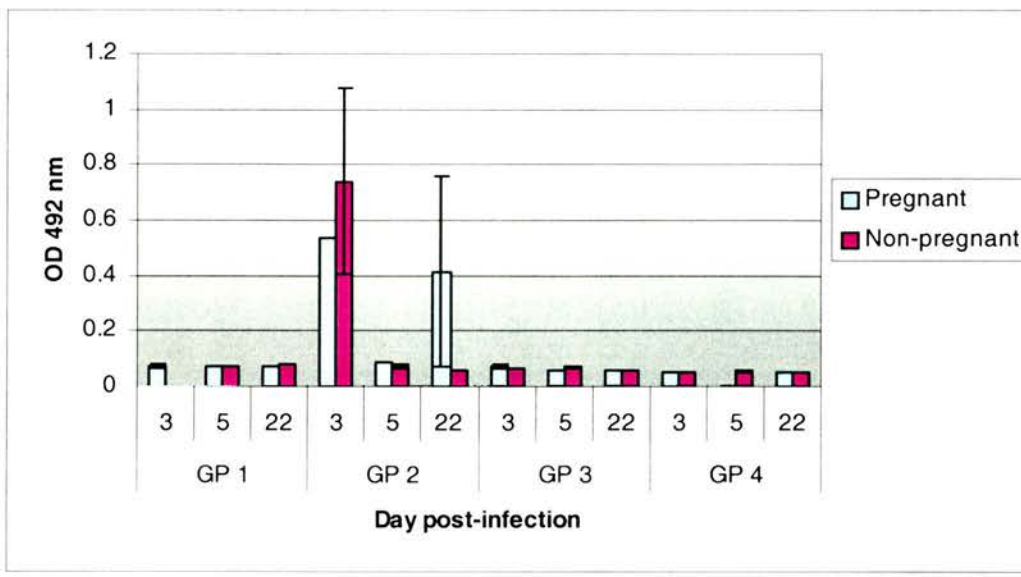
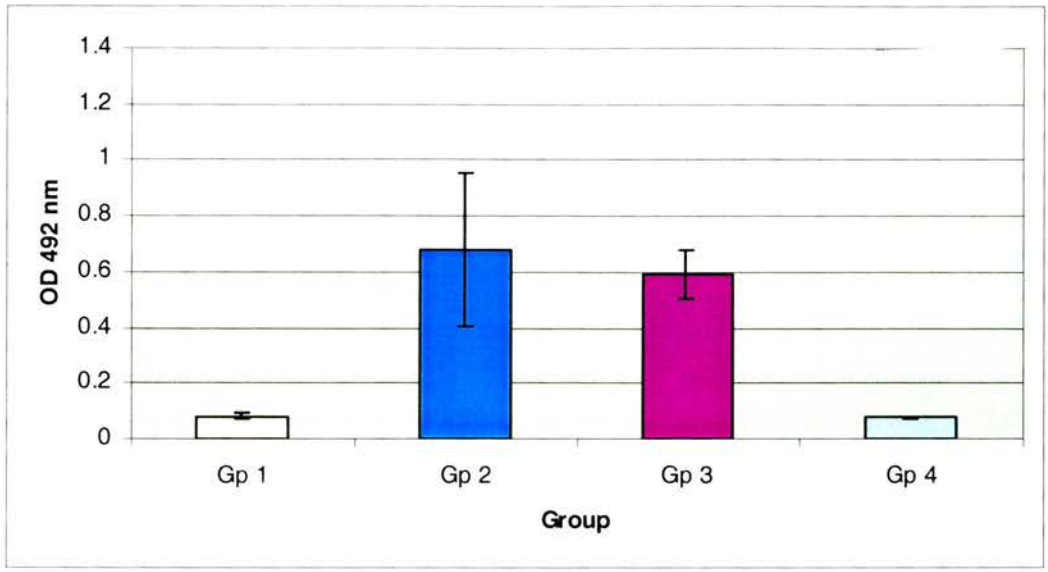


Figure 6.9 IgG1 antibody response in control and infected mice (a) post-primary inoculation/pregnancy. Mean (± SEM) ODs were determined by ELISA for all mice (pregnant and non-pregnant) in each group on day 40 post-primary inoculation. **(b) post-secondary inoculation/pregnancy.** Mean (± SEM) ODs were determined by ELISA for each group of pregnant and non-pregnant mice on each day p.i. (the final PM day post-second infection). NB. No non-pregnant mice were present in group 1 on day 3 p.i, and no pregnant mice in group 4 on day 5 p.i. Where there are no error bars present, one mouse only was killed at that time point (**Table 6.3**).

The IgG2a antibody response to *C. abortus* was significantly higher in the infected groups (2 and 3) compared to the control groups (1 and 4) after the first pregnancy ($F_{3,36}$=7.77, p<0.001) (**Figure 6.10a**). IgG2a responses, which are indicative of a Th1 immune response, continued to increase after the second pregnancy in groups 2 and 3, following inoculation with PBS (group 2) or secondary infection (group 3) (**Figure 6.10b**). Higher IgG2a levels were detected in sera from group 2 than group 3 mice, despite the lack of secondary challenge in this group 2. Comparison of IgG2a responses in pregnant and non-pregnant mice revealed no significant differences ($F_{1,36}$=0.49, p=0.491), although higher IgG2a responses were observed in non-pregnant mice of all groups, except on day 5 p.i. (group 2) and day 22 p.i. (group 3). IgG2a responses remained low in sera from the inoculum control mice (group 4), although the titres had started to increase by day 22 p.i.. Significantly higher responses were determined in groups 2 and 3 than in groups 4 and 1 ($F_{3,36}$=4.99, p=0.01) after the second pregnancy.

(a)



(b)

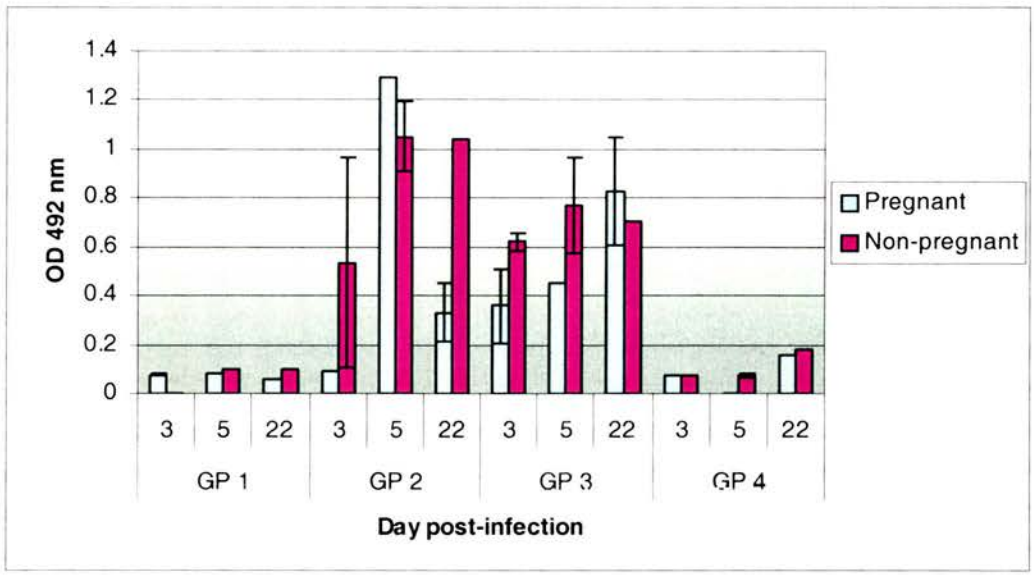
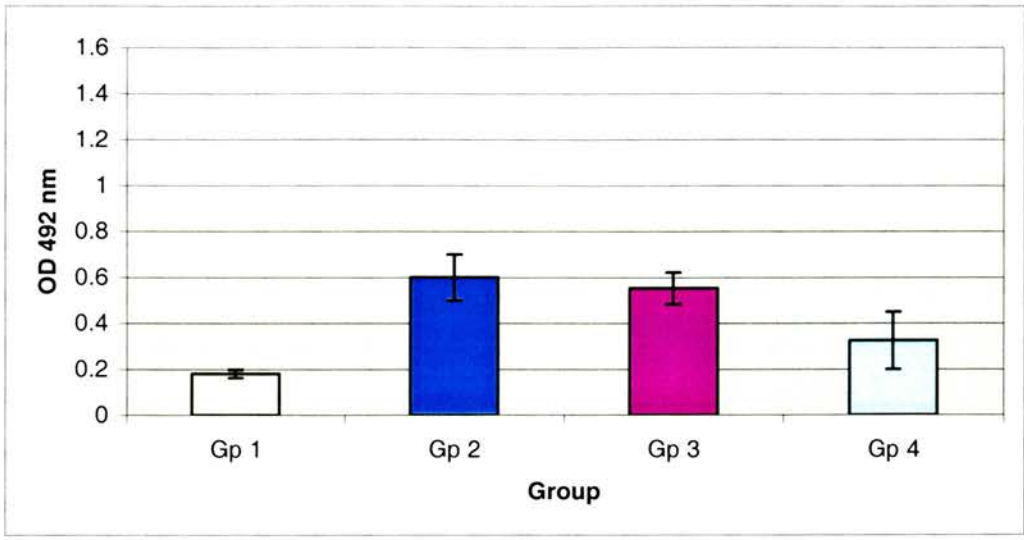


Figure 6.10 IgG2a antibody response in control and infected mice (a) post-primary inoculation/pregnancy. Mean (± SEM) ODs were determined by ELISA for all (pregnant and non-pregnant) mice in each group on day 40 post-primary inoculation (between the first and second pregnancies). **(b) post-secondary inoculation/pregnancy.** Mean (± SEM) ODs were determined by ELISA for each group of pregnant and non-pregnant mice on each day p.i. (the final PM day post-second infection). NB. No non-pregnant mice were present in group 1 on day 3 p.i, and no pregnant mice in group 4 on day 5 p.i. Where there are no error bars present, one mouse only was killed at that time point (**Table 6.3**).

IgM titres were significantly higher in the infected groups (2 and 3) compared with the control groups (1 and 4) after the first pregnancy/inoculation ($F_{3,36}$=5.50, p=0.004) (**Figure 6.11a**), similar to the IgG2a titres. IgM titres varied significantly throughout infection in the groups after the second pregnancy being higher in all infected (groups 2-4) than in the uninfected (group 1) mice ($F_{3,36}$=4.05, p=0.021) (**Figure 6.11b**). Few differences were observed between IgM responses in sera from group 2 and 3 mice, and these responses were similar to those in sera from group 4 mice. IgM responses varied significantly during infection, with a higher titre detected on days 5 and 22 p.i. than on day 3 p.i. in infected groups 2, 3 and 4 ($F_{2,36}$=4.18, p=0.030). As with IgG1 and IgG2a responses, the IgM titre was not significantly different in pregnant compared to non-pregnant mice ($F_{1,36}$=0.18, p=0.675), although the majority of non-pregnant mice had higher responses, with the exception of groups 2 and 4 on day 22 p.i. and group 3 on day 5 p.i..

(a)



(b)

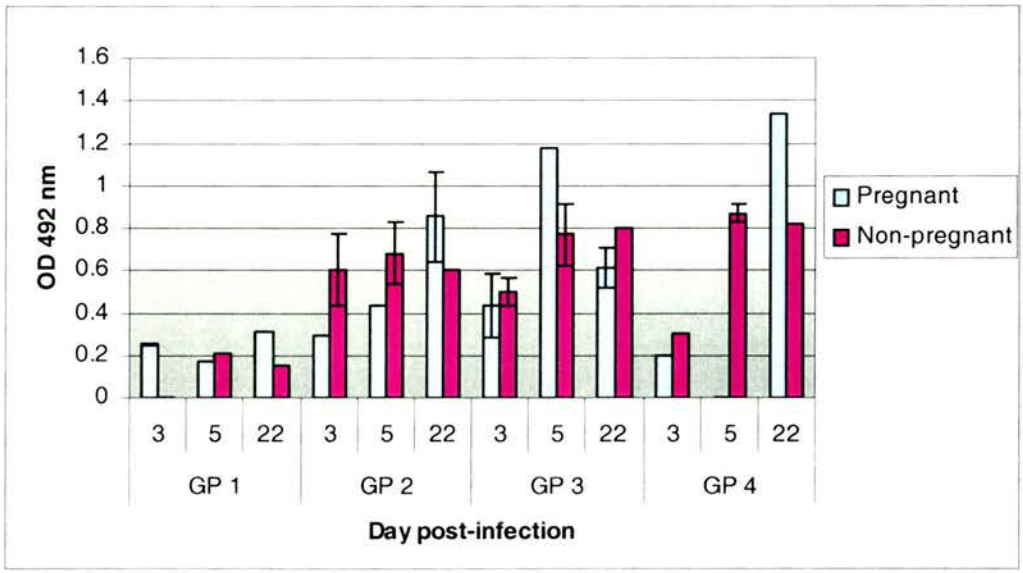


Figure 6.11 IgM antibody response in control and infected mice (a) post-primary inoculation/pregnancy. Mean (± SEM) ODs were determined by ELISA for all (pregnant and non-pregnant) mice in each group on day 40 post-primary inoculation (between the first and second pregnancies). **(b) post-secondary inoculation/pregnancy.** Mean (± SEM) ODs were determined by ELISA for each group of pregnant and non-pregnant mice on each day p.i. (the final PM day post-second infection). NB. No non-pregnant mice were present in group 1 on day 3 p.i, and no pregnant mice in group 4 on day 5 p.i. Where there are no error bars present, one mouse only was killed at that time point.

6.5.5.2 Western blotting

To identify the specificities of the antibody responses, IgG1 and IgG2a responses to *C. abortus* EBs were assessed by Western blot analysis, as described in **Section 2.9.2**. **Figure 6.12a** shows representative results of the almost undetectable IgG1 response against whole EBs. This contrasts with the high IgG1 response of group 2 detected by ELISA in response to whole EBs. There are only faint bands when hyperimmune sera from *C. abortus*-infected mice was used, at approximately 90 kDa.

Anti-IgG2a reactivity to whole EBs (**Figure 6.12b** and **c**) was notably higher than the IgG1 reactivity when mouse hyperimmune sera was used. Serum samples in lanes 3 and 4 in **Figure 6.12b** represent sera from uninfected mice (group 1), showing that there was no response in comparison to infected mice. Serum samples in lanes 5, 7-12 and 14, representing group 2 mice, showed a strong IgG2a response to a number of proteins (**Figure 6.12b**). Similarly sera from group 3 (lanes 2-12; **Figure 6.12c**) reacted to a wide range of proteins, whereas this reactivity is reduced in group 4 sera (lanes 13 and 14, **Figure 6.12c**). Although MOMP is the major immunodominant antigen, sera did not appear to react specifically to MOMP but to a range of proteins, at approximately 90, 60 and 25 kDa.

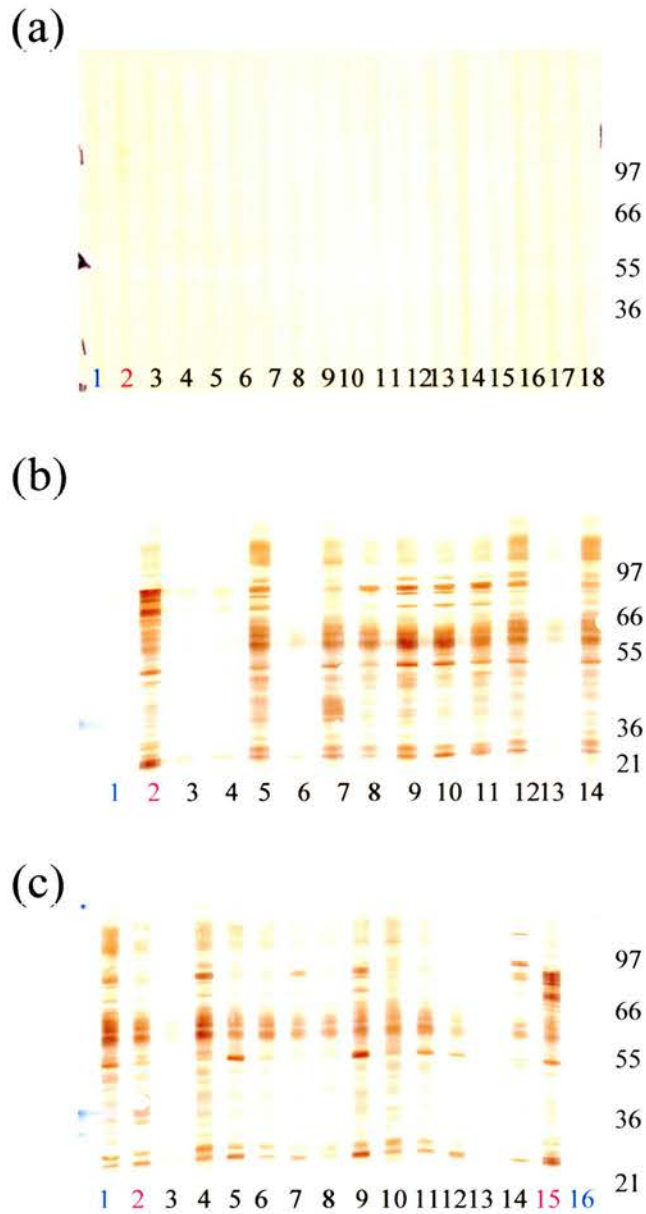


Figure 6.12 IgG1 and IgG2a responses in sera from control and infected mice post-secondary inoculation to EBs by Western blots analysis. (a) IgG1 response : 1 negative control, 2 mouse hyperimmune sera, **3-4** negative control mice, **5-9** group 2 sera, **10-14** group 3 sera, **15-18** group 4 sera. **(b) IgG2a response:** 1 negative control, 2 mouse hyperimmune sera, **3-4** negative control mice, **5-14** group 2 sera. **(c) IgG2a response: 1** group 2 serum, **2-12** group 3 sera, **13-14** group 4 sera, 15 mouse hyperimmune sera, 16 negative control.

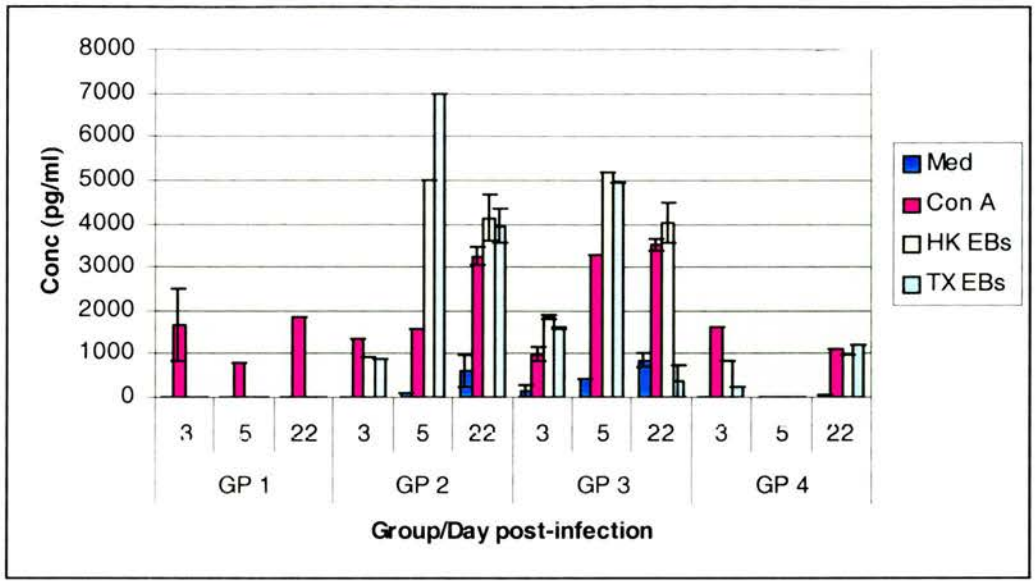
6.5.6 Cytokine production during *C. abortus* infections

6.5.6.1 IFN-γ protein expression

IFN-γ concentrations in supernatants from splenocytes stimulated with specific *C. abortus* antigens were determined, as described in **Sections 2.10** and **2.14**. Although no statistically significant different results were observed between pregnant and non-pregnant mice ($p>0.1$ for all antigens), IFN-γ concentrations appeared consistently higher in non-pregnant mice (**Figure 6.13b**).

IFN-γ was produced specifically in response to *C. abortus* HK and TX EBs by *in vitro* stimulation of splenocytes from mice that had been infected with *C. abortus* (groups 2, 3 and 4) (**Figure 6.13**). Generally, HK EBs induced greater IFN-γ production than TX EBs, although overall there were little differences. Very low concentrations of IFN-γ were detected in response to medium alone, although this was slightly elevated in splenocytes of infected groups 2 and 3. IFN-γ responses were significantly higher in HK ($F_{3,36}=9.46$, $p<0.001$) and TX ($F_{3,36}=8.39$, $p<0.001$) EB-stimulated splenocytes from groups 2 and 3 than in those of group 4. An increasing trend in IFN-γ concentration was observed from day 3 to 5 p.i. in pregnant mice of groups 2 and 3 in response to HK and TX EBs (**Figure 6.13a**). Concentrations remained high on day 22 p.i. in pregnant mice of groups 2 and 3 and a slight increase was also detected from day 3 to 22 p.i. in group 4 (**Figure 6.13a**). A similar increasing trend was observed over the course of infection in non-pregnant mice of groups 2 and 4, whereas in non-pregnant mice of group 3, similar responses were detected on all of days 3, 5 and 22 p.i. (**Figure 6.13b**). These slight differences in cytokine production over the course of infection (in pregnant and non-pregnant mice) were not significant in response to either HK ($F_{2,36}=1.08$, $p=0.359$) or TX EBs ($F_{2,36}=2.44$, $p=0.113$).

(a)



(b)

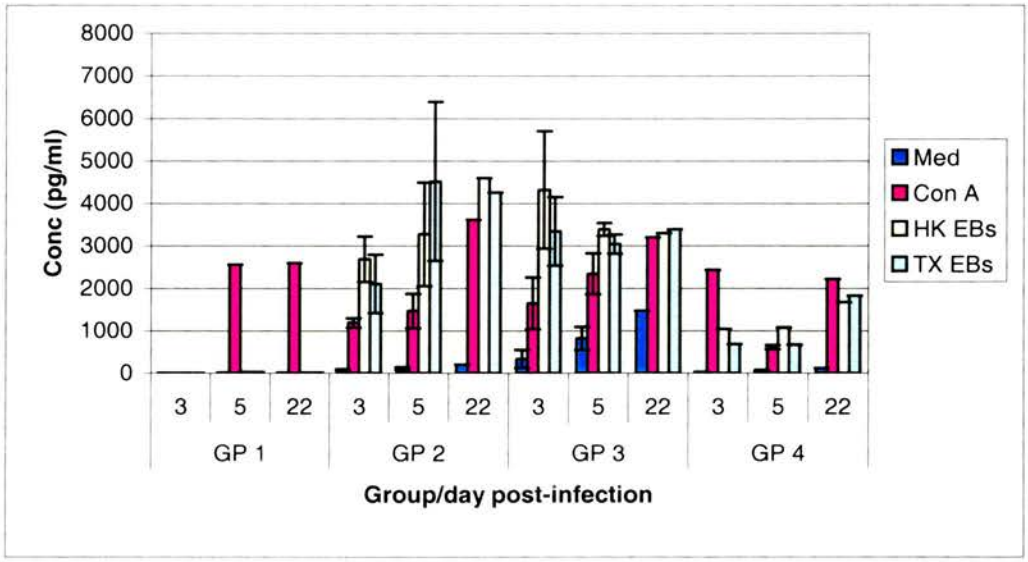


Figure 6.13 Splenic IFN-γ concentrations as a result of *in vitro* stimulation with specific *C. abortus*-antigens in control and infected mice after secondary inoculation (a) pregnant mice and (b) non-pregnant mice. Data are presented as means (± SEM) for each group of mice on days 3, 5 and 22 p.i, determined by commercial ELISA using known standards (**Section 2.10**). Medium=RPMI Complete, Con A= 1.25 µg, HK EBs= 0.6 µg and TX EBs= 0.6 µg.

6.5.6.2 TNF-α protein expression

TNF-α, another proinflammatory Th1 cytokine, expression was also monitored in response to specific chlamydial antigen stimulation (**Figure 6.14**), as described in **Sections 2.10** and **2.14**. Representative samples were analysed to give an indication of the levels of TNF-α production in uninfected and infected mice.

Similar to the IFN-γ response (**Figure 6.13**), there was little cytokine production to medium alone compared to the positive control (Con A) and to specific chlamydial antigens (**Figure 6.14**). No obvious differences were observed in the TNF-α response between pregnant and non-pregnant mice, however this could not be analysed for statistical significance due to the low sample number. TNF-α production was highest in infected mice (groups 2, 3 and 4) in response to *C. abortus*-specific antigens and HK EB-induced TNF-α responses were almost consistently higher than those induced by TX EBs. Between 500-1000 pg/ml (866.9 $\pm$ 120) TNF-α was produced by stimulated cells in group 4 (in response to EBs) and higher levels of 600-1500 pg/ml (1037.3 $\pm$ 202) and 500-2000 pg/ml (1161.9 $\pm$ 165) were produced by stimulated cells in groups 2 and 3, respectively, showing that there was little difference between the two latter immune groups. TNF-α concentrations were lower than IFN-γ (**Figure 6.14**) in the positive control and EB-specific supernatants, with maximum levels of 2000 and 5000 pg/ml, for TNF-α and IFN-γ, respectively.

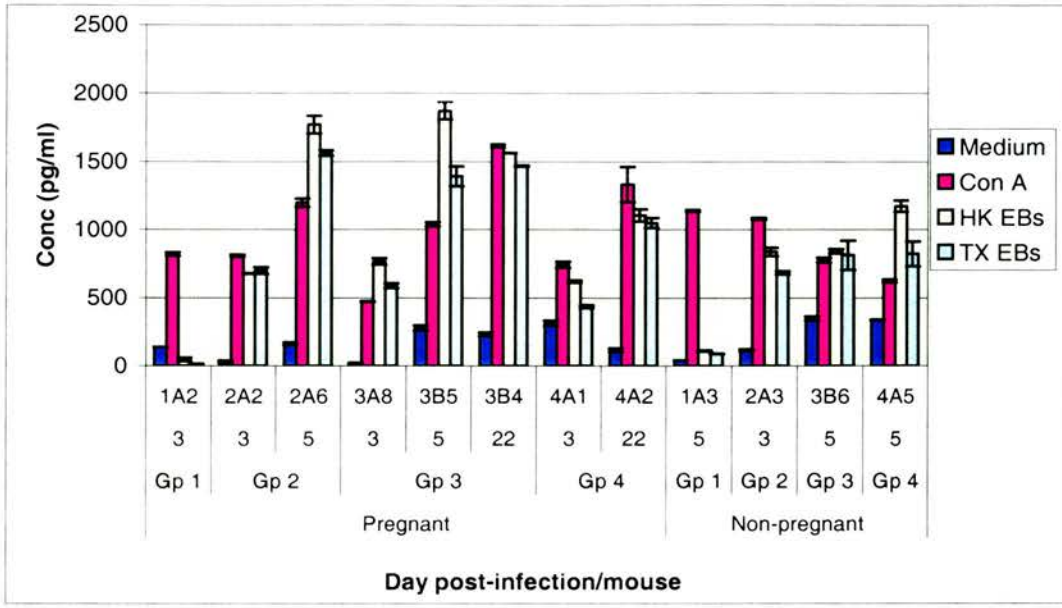


Figure 6.14 TNF-α concentrations as a result of *in vitro* stimulation of splenocytes with specific *C. abortus*-antigens from pregnant and non-pregnant control and infected mice. Data are presented as means (± SD), determined by commercial ELISA for representative mice from each group. Prefix 1,2, 3 or 4 signifies the group.

Representative serum samples (collected at the PM) were also analysed for TNF-α production by commercial ELISA, as shown in **Table 6.5**. Despite some of the mice mentioned in **Table 6.5** inducing TNF-α expression in response to specific chlamydial antigens in supernatants collected from stimulated splenocytes and having a detectable background response to medium alone (**Figure 6.14**), no TNF-α was detected in these serum samples. TNF-α was identified in sera from group 4 mice, although concentrations were extremely low in comparison to those detected in cell supernatants in response to specific chlamydial antigens. These differences in cytokine concentrations in supernatants and sera may be due to some inhibitory substances in sera preventing identification of TNF-α. It also suggests that cytokine concentrations in sera are unrepresentative of the local immune responses within infected tissues.

Table 6.5 TNF-α concentrations in serum samples after the second pregnancy

Mouse	Day p.i.	Conc (pg/ml)
2A6	5	0
2A3	3	0
3A8	3	0
3A2	3	0
3B6	5	0
4A1	3	65.2
4A6	5	53
4A5	5	27.17
4A3	3	0

Prefix 2,3 or 4 denotes the group (**Table 6.1**).

6.5.7 *In situ* hybridisation

Having determined the protein expression of the proinflammatory cytokines, IFN-γ and TNF-α, mRNA expression in tissues was examined by *in situ* hybridisation, as described in **Section 2.11.5**. IFN-γ and TNF-α mRNA$^+$ cell counts are shown in **Tables 6.6** and **6.7**, respectively.

A lower number of IFN-γ mRNA$^+$ cells were detected in this experiment (**Table 6.6**) than in primary-infected pregnant mice in **Chapter 4**. All sections from uninfected mice (group 1) were negative. One aborted placenta collected after the first pregnancy from group 2 had IFN-γ$^+$ cells present but no mRNA expression of IFN-γ was detected in any liver or placental tissues from this group following the second pregnancy. IFN-γ mRNA$^+$ cells were also observed in aborted placenta tissue collected after the first infection/pregnancy in group 3, whereas no positive cells were present in placental tissue following secondary infection. Low numbers of cells expressing IFN-γ mRNA were detected on days 3 and 5 p.i. in liver sections of immune mice (group 3), and one mouse (3B6) in particular had very high numbers of positive cells compared to liver sections from mice of the same group (**Table 6.6**). Numerous IFN-γ$^+$ cells were present in liver tissue from inoculum control mice (group 4), following their primary infection with *C. abortus* in the second pregnancy.

Interestingly, positive cells were detected in the placenta of one mouse from this group (4A1), but not in the liver of the same mouse on day 3 p.i., suggesting IFN-γ may be localised to sites of infection. This is supported by culture (**Section 6.5.2**) and immunohistochemistry data (**Section 6.5.3**). IFN-γ mRNA$^+$ expression had decreased in group 4 by day 22 p.i., coinciding with the lack of detectable infection by culture and immunohistochemistry.

Table 6.6 IFN-γ counts in tissue samples from control and infected mice after the first and second pregnancies

	Mouse	Tissue	Day p.i.	Cells/mm^2
Pregnancy 1	2A2	Aborted Placenta	3	6.5
	3A2		3	1.45
	3B6		5	2.9
Pregnancy 2 Pregnant mice	3B6	Liver	5	11.6
	4A1	Placenta	3	4.35
	4A2	Liver	22	1.45
Pregnancy 2 Non-pregnant mice	3A5	Liver	3	2.9
	3A3	Liver	3	1.45
	3A4	Liver	5	1.45
	3B3	Liver	5	4.35
	4A3	Liver	3	5.8
	4A5	Liver	5	4.8
	4A5	Liver	5	6.1

Numbers of positive cells/mm^2 is shown for each mouse for each of liver and placenta tissue at each time point post-secondary inoculation. Prefix 2, 3 or 4 denotes the group (**Table 6.1**).

TNF-α mRNA$^+$ cells were detected in only 5 of 38 liver sections examined, 4 of which were from the inoculum control (group 4) mice, the fifth infected in the first pregnancy only (group 2) (**Table 6.7**). TNF-α$^+$ cells were observed only in infected liver tissue sections, and were fewer in number than IFN-γ$^+$ cells.

Table 6.7 TNF-α mRNA$^+$ cells in liver tissue after the second pregnancy

Mouse	Day p.i.	Cell/mm^2
2A6	5	2.9
4A1	3	1.4
4A6	5	2.9
4A4	22	1.4
4A2	22	2.9

Prefix 2 or 4 denotes the group (**Table 6.1**).

6.5.8 LSAs

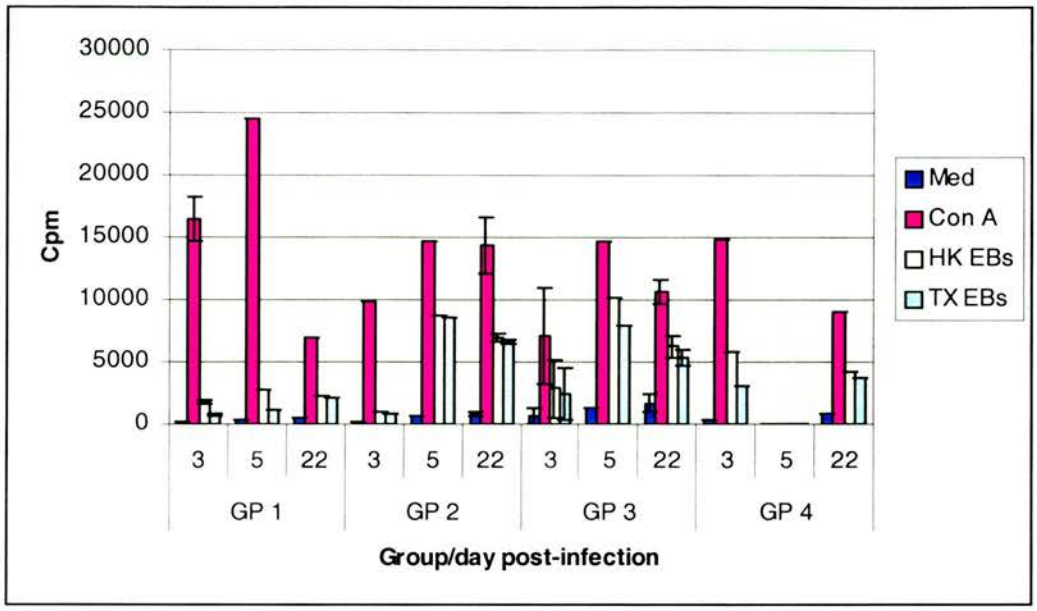
To investigate stimulation of mouse splenocytes in response to specific chlamydial-antigens, HK and TX EBs, LSAs were carried out, as described in **Section 2.13**. In order to demonstrate the wide variation associated with this assay, absolute counts/min (**Figure 6.15**) and stimulation indices (SIs; **Figure 6.16**) are presented for both pregnant and non-pregnant mice although there were no significant differences between the two groups of mice ($p>0.1$ for all antigens).

C. abortus-specific responses were detected in all infected mice (**Figure 6.15** and **6.16**). An increased proliferation (counts/min; cpm) to both HK and TX EBs was observed from day 3 to 5 p.i., with a slight decrease in proliferation on day 22 p.i. in pregnant mice that were immune to abortion (groups 2 and 3), with similar responses obtained in these two groups (**Figure 6.15a**). Examination of SIs of the same data from pregnant mice revealed a less pronounced increase from day 3 to 5 p.i. in group 2, and the SIs appeared similar at all time points in group 3 (**Figure 6.16a**).

Similar proliferative responses (cpm) were observed throughout infection in non-pregnant mice, with a decrease to both HK and TX EBs on day 22 p.i. in groups 2 and 3 (**Figure 6.15b**). In contrast, SIs imply that proliferation increased rather than decreased in response to both HK and TX EBs on day 22 p.i. in group 3 mice (**Figure 6.16b**). Responses varied slightly in group 4 and were lower than those of groups 2 and 3 throughout the infection (**Figure 6.15b**).

There were significant differences in proliferative responses to both HK ($F_{3,36}$=12.34, p<0.001) and TX EBs ($F_{3,36}$=12.54, p<0.001) between the groups of mice (pregnant and non-pregnant), being higher in groups 2 and 3 than 1 and 4, in a similar trend to IFN-γ production. HK EB-induced stimulation was significantly ($F_{2,36}$=4.22, p=0.024) higher on day 5 p.i. in all groups of mice, whereas stimulation induced by TX EBs was similar throughout infections ($F_{2,36}$=0.48, p=0.625).

(a)



(b)

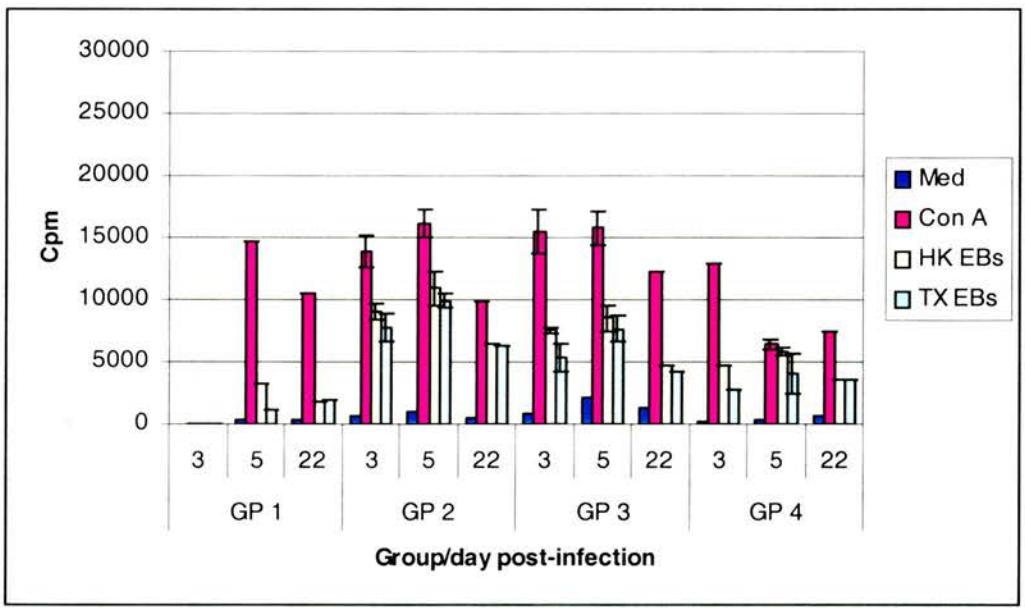
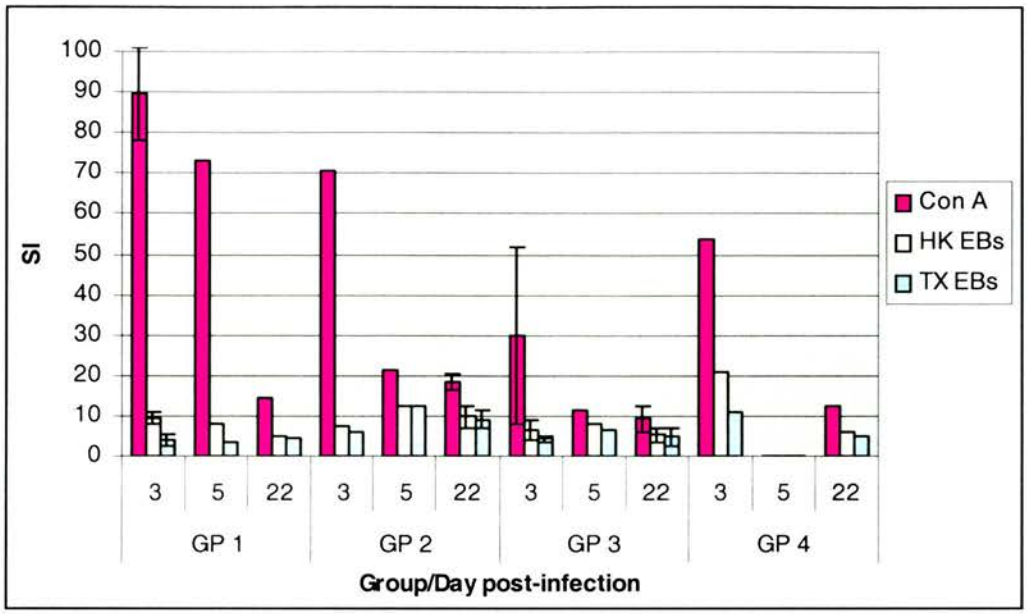


Figure 6.15 Proliferation induced by specific chlamydial antigens of splenocytes from control and *C. abortus*-infected (a) pregnant and (b) non-pregnant mice, detected by incorporation of ^{3}H-thymidine. Results are shown as absolute cpm values calculated from the arithmetic means of triplicate measurements for each group on each day p.i.. The stimulating antigens were: Medium (background control; RPMI Complete), Con A (1.25 µg), HK EBs (0.6 µg) and TX EBs (0.6 µg).

(a)



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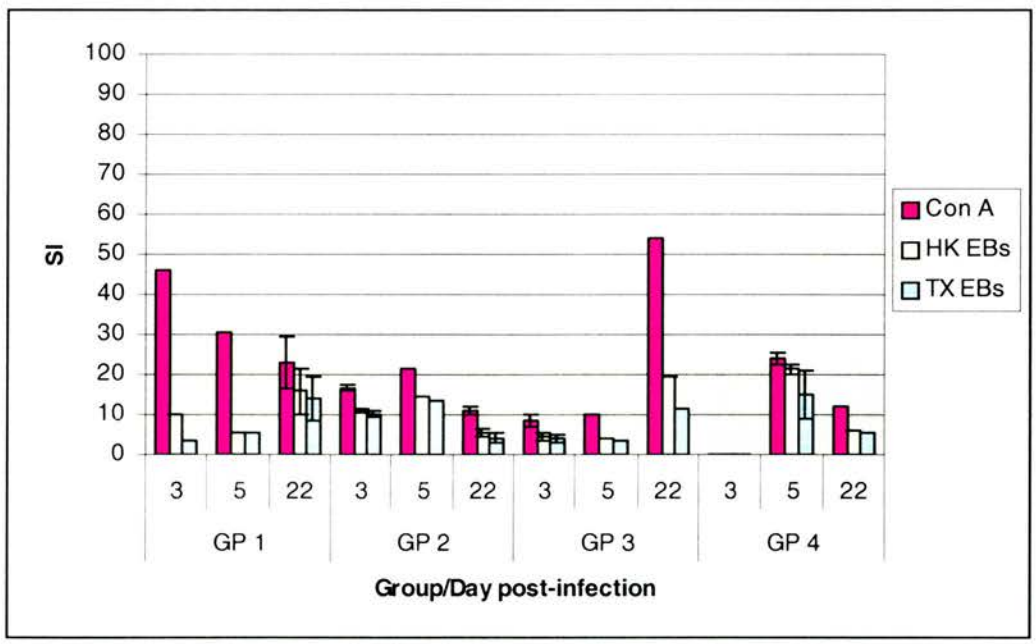


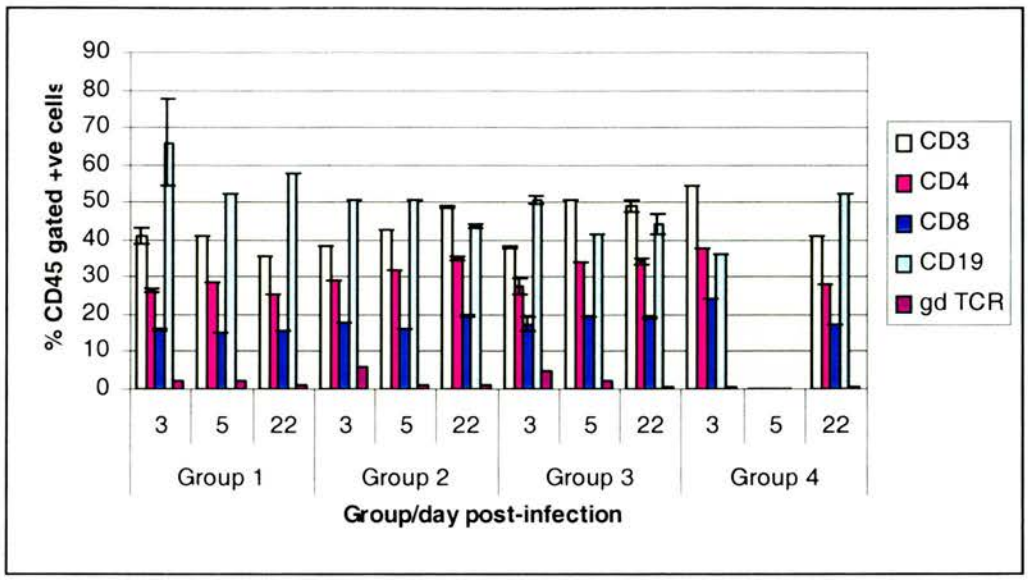
Figure 6.16 Stimulation indices of proliferation induced by specific chlamydial antigens of splenocytes from control and *C. abortus*-infected (a) pregnant and (b) non-pregnant mice, detected by incorporation of ^{3}H-thymidine. Results are shown as mean (± SEM) SIs calculated from the arithmetic means of triplicate measurements in relation to the background cpm for each group on each day p.i.. The stimulating antigens were: Con A (1.25 µg), HK EBs (0.6 µg) and TX EBs (0.6 µg).

6.5.9 Flow cytometry analysis

Flow cytometry analysis was used (**Section 2.15**) to identify cell subpopulations expressed in the different control and infected groups of mice (**Table 6.1**). Gates for flow cytometry analysis were set to $CD45^+$ cells (**Figure 6.17**).

No clear observable trends were observed in any of the cell populations determined between the groups of mice (**Figure 6.17**). The percentage of $CD3^+$ cells was significantly higher on days 5 and 22 p.i. in pregnant mice than non-pregnant mice, with little difference between the two groups of mice on day 3 p.i. ($F_{2,34}$=3.75, p=0.043). Percentages of $CD3^+$ cells were similar in all groups of mice. As with $CD3^+$ cells, more $CD4^+$ cells were present in pregnant mice than non-pregnant mice ($F_{1,34}$=18.05, p<0.001) and percentages of $CD4^+$ T cells were also similar in all groups of mice. Pregnant mice had a higher percentage of $CD8^+$ T cells than non-pregnant mice and this varied significantly between the groups of mice ($F_{3,34}$=3.20, p=0.048), with slightly more $CD8^+$ T cells detected in group 4 pregnant mice. Very low numbers of γδ T cells were detected in all mice and this population was also significantly higher in pregnant than non-pregnant mice ($F_{3,32}$=3.20, p=0.048). In contrast to the T cells, significantly more $CD19^+$ cells (B cells) were identified in non-pregnant than pregnant mice ($F_{1,34}$=12.87, p=0.002). $CD19^+$ cells were the predominant cell type in all groups of mice, with highest numbers detected in uninfected mice.

(a)



(b)

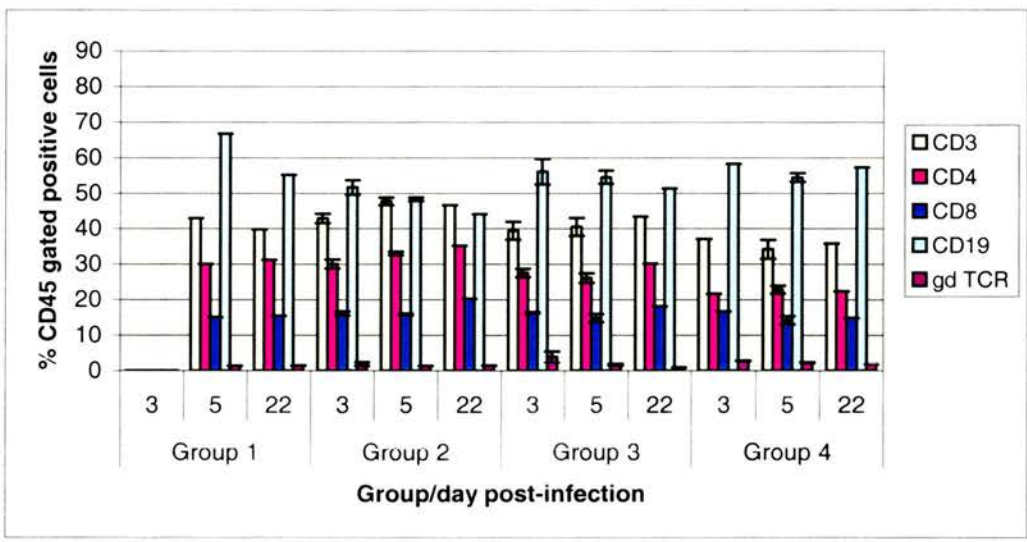


Figure 6.17 Phenotypic variation in splenocyte subpopulations of (a) pregnant and (b) non-pregnant control and infected mice. Analysis was performed on CD45 gated cells. Mean (± SEM) percentages for each cell type for each group on the different days p.i. are presented. gd = γδ TCR.

6.6 Discussion

This pregnant mouse model of chlamydial abortion has similarities with ovine chlamydial infections, including abortion and induction of a Th1 type immune response. Although these similarities verify the use of the pregnant mouse model for assessing vaccines against EAE, it was important to compare other aspects of ovine chlamydial infections to the disease in mouse to fully validate the model system. The purpose of the study described in this chapter was to examine immunity to abortion in mice, for comparison with immunity to abortion in sheep, in the following pregnancy post-abortion. The model was used to determine if, following primary infection and abortion, mice would be immune to abortion in the subsequent pregnancy with (group 3) and without (group 2) experimental secondary infection. For the remainder of this chapter, the groups shall be referred to as the following, with 'P' defining pregnancy: Group 1 (P/P) mice were inoculated with PBS only in both the first and second pregnancies, i.e. uninfected; Group 2 (1°P/P) mice were infected with *C. abortus* in the first pregnancy and PBS in the second; Group 3 (1°P/2°P) mice were inoculated with *C. abortus* in both pregnancies i.e. primary and secondary infections; and group 4 (P/1°P) mice were inoculated with PBS in the first pregnancy and a primary infection of *C. abortus* in the second pregnancy.

Group 2 (1°P/P) and 3 (1°P/2°P) mice that were infected with *C. abortus* at mid-gestation aborted as expected in the first pregnancy and groups 1 (P/P) and 4 (P/1°P) littered live healthy pups. The pregnancy rate decreased in the second pregnancy, which may have been due to the previous infection affecting the successful impregnation of the mice, as possibly occurred in a previous study (**Chapter 5**). Although this could have been a result of the primary infection with *C. abortus* affecting the fertility of the mice, it is unlikely, as a similar pattern would not have occurred in groups 1 (P/P) and 4 (P/1°P), which were infected only with PBS in the first pregnancy. It would also contrast to what occurs in sheep, where those ewes that have aborted can continue to lamb successfully in subsequent breeding seasons. Regardless of the lower pregnancy rate, outcomes of infection for each group were determined in the second pregnancy. Uninfected mice (group 1; (P/P)) also littered in

the second pregnancy, and mice in the inoculum control group 4 (P/1°P) were successfully infected with *C. abortus*, demonstrated by the recovery of live organisms from tissues and abortion.

Mice that aborted in the first pregnancy but were mated again without secondary experimental infection (group 2; 1°P/P), produced live, healthy pups mimicking the immunity that occurs in sheep following natural and experimental primary infections (Papp *et al.*, 1994; Aitken & Longbottom, 2004). It can be concluded that disease in the mouse model is analogous to that in sheep in terms of immunity to abortion in a subsequent pregnancy, and this is further supported by group 3 (1°P/2°P) animals that aborted in the first pregnancy and were mated again. These mice were subsequently inoculated with a secondary infection of *C. abortus* and this resulted in littering of live, healthy pups demonstrating immunity to secondary infection, which has also been shown in sheep (Papp *et al.*, 1994). It is probable that sheep may be infected again around the time of lambing, particularly if it is in the second breeding season after *C. abortus* entered the flock. One problem associated with routinely using sheep that are immune to abortion for breeding is the likelihood that a chronic reproductive tract infection has developed, as has been demonstrated by Papp *et al.* (Papp *et al.*, 1994, 1998). Ewes that had previously aborted were found to excrete *C. abortus* organisms at the time of oestrus although this was in a very small sample number (Papp *et al.*, 1994, 1998). This implies that other ewes could be infected at this time, showing that it is important to segregate previously aborted ewes at oestrus and lambing to prevent other flock members becoming infected. It would have been useful to take vaginal swabs after the first and second pregnancies in mice to determine if shedding occurs, as this may have provided further information on sterility, of which little is known in the ovine field.

Having identified that immunity to abortion occurred in groups 2 (1°P/P) and 3 (1°P/2°), the incidence of *C. abortus* and the nature of the immune responses were examined. Chlamydial organisms were recovered and chlamydial antigen was detected only in mice that were infected in the second pregnancy (group 4; P/1°P) until day 5 p.i.. *C. abortus* was not detected in any tissues from either of the group 2

(1°P/P) or 3 (1°P/2°P) animals that were immune to abortion. This indicates that the animals had cleared the infection or the level of infection was too low to be detected in pregnant mice of group 2 and there was no recrudesce to cause abortion. No organisms were detected in non-pregnant mice of groups 2 or 3. Adaptive immunity was present in group 3 mice, shown by control of the secondary infection, as denoted by the lack of detection of antigen and organisms from day 3 p.i. and also by the lack of abortion. Therefore immunity appears to be sterile although this was only determined by culture and immunohistochemistry. PCRs (**Chapter 5**) could be adopted in future studies in both mice and sheep to monitor *C. abortus* infection post-abortion.

The inflammatory response agreed with the recovery and antigen data, being significantly higher in tissues from inoculum control mice (group 4; P/1°P) than from tissues of group 2 (1°P/P) and 3 (1°P/2°P) mice, suggesting that there were either no or very few organisms present. The lack of organisms is likely to be a result of control by the acquired immune response in group 3 rather than that of innate immunity in group 4, therefore there was less inflammation in groups 2 and 3 than in group 4 mice. It should be noted that PMN infiltration had also decreased by day 22 p.i. (approximately 14 days post-abortion) in tissues from group 4 (P/1°P) mice, coinciding with a reduction/clearance of organisms and a change from innate to cell mediated immunity. Papp and Shewen (1996) reported that there was no evidence of pathology associated with persistent infections in the uterus of sheep that were immune to abortion, but plasma cells and intraepithelial lymphocytes were increased in number and distribution. Uteri from some mice across all infected groups were macroscopically enlarged and reddened. This could have been as a result of increased inflammation, as demonstrated by detection of PMNs, B cells, $CD4^+$ and $CD8^+$ T cells in the uterine epithelium or purely related to the impact of pregnancy. However both PMNs and B cells are suggested to have limited roles in immunity to secondary infections of *C. abortus* (Buendia *et al.*, 2002; de Oca *et al.*, 2000).

NK cells were not detected in any of the tissues analysed at the University of Murcia. In contrast, Buendia *et al.* (Buendia *et al.*, 2004) have shown that NK cells increase

in liver and spleen of non-pregnant mice in response to early *C. abortus* infection, where NK-depleted mice were shown to be more susceptible to infection, as demonstrated by higher mortality and infection levels. Differences between this study and that by Buendia include the pregnancy status of the mice and the period of time between infection and assessment for NK cell presence. Since NK cells may only have a role in the early innate response, this explains their absence in immune mice (groups 2 (1°P/P) and 3 (1°P/2°P)) but not in mice from group 4 (P/1°P), although the time scales were different.

A Th1 cell mediated response controls *C. abortus* infections (de Oca *et al.*, 2000; Del Rio *et al.*, 2000). Characterisation of the T cell response in the present study identified both $CD4^+$ and $CD8^+$ T cells in infected liver and placenta tissues in mice across all groups. Higher numbers of $CD4^+$ T cells, which are characteristic of a Th1 immune response, were identified than $CD8^+$ T cells in all mice throughout infection, a finding similar to that observed in ovine infections (Buxton *et al.*, 2002). No distinct differences were detected between infected groups or pregnant and non-pregnant mice, and a $CD4^+$ T cell response was elicited in all infected mice (groups 2 (1°P/P), 3 (1°P/2°P) and 4 (P/1°P)). It may have been expected that T cell numbers would be higher in group 3 mice compared to group 4, due to the acquired immune response. $CD4^+$ and $CD8^+$ T cell numbers were widely variable in T cell number and distribution within liver sections in all groups and placental tissue from immune mice (groups 2 (1°P/P), 3 (1°P/2°P)), which may indicate individual differences in immunity. $CD4^+$ and $CD8^+$ T cells have been identified in infected liver sections in other mouse models of *C. abortus* (de Oca *et al.*, 2000; Del Rio *et al.*, 2001), showing that similar immune responses are mounted despite differences in mouse strains and *C. abortus* isolates. Immunity to secondary infection with *C. trachomatis* depends on $CD4^+$ T cells (Williams *et al.*, 1997), and as demonstrated in this study, higher numbers of $CD4^+$ cells were detected than $CD8^+$ cells. It has been claimed that $CD8^+$ cells may be more important in the resolution of *C. abortus* infections (Del Rio *et al.*, 2001; de Oca *et al.*, 2000), which does not agree with this study.

Very few differences were identified in cell subpopulations by flow cytometry in infected mice compared to uninfected mice. No differences were even detected between cell subpopulations in infected and uninfected mice by searching for blasting cells, based on size. B cells were more numerous in all mice than T cells, and CD19 cells were higher in uninfected mice (group 1; P/P) than all infected mice (groups 2-4), although these differences were not significant. T cell numbers may have been expected to increase in immune mice (group 3) but this was not observed. Differences may have been observed in lymph nodes more localised to sites of infection, such as the uterine lymph node, as unless the infection remains in the spleen, it is difficult to detect differing cell subpopulations in response to a circulating infection. Due to their small size, uterine lymph nodes would need to be pulled for each group in order to obtain sufficient cells for flow cytometry analysis. Slightly more $CD4^+$ than $CD8^+$ T cells were detected in the spleen and numbers of γδ T cells was particularly low in all groups of mice, as expected (REF). It is suggested that αβ T cells are the major T cell population in acquired immunity to chlamydial infection and that γδ T cells play an ancillary role in regulating the αβ T cell response (Yang *et al.*, 1998). These γδ T cells may be important early in infection, as observed in models of *Salmonella* and trypanosome infections (Williams *et al.*, 1996).

Unexpectedly, IgG1 titres were extremely high in sera from group 2 (1°P/P) mice compared to the other groups of infected mice and previous studies (**Chapter 4**). IgG1 is indicative of a Th2 response therefore it was not expected to increase in *C. abortus*-infected mice (group 2; 1°P/P), as *C. abortus* infections are controlled by a Th1 type immune response (**Chapter 4**; Buendia *et al.*, 2004). However, a similar effect was not observed after the primary infection in group 3 and these two groups of mice were infected with exactly the same inoculum. Wide variation exists in group 2 (1°P/P) animals, as demonstrated by the large error bars, therefore the high IgG1 response was not observed in all mice of this group, in 5 out of 12 mice. The majority, but not all, of the mice that had a high IgG1 response were pregnant, therefore it could have been related to the more dominant Th2 response that is thought to be prevalent during pregnancy (Kidd, 2003), however, the low IgG1 response of pregnant and non-pregnant mice in group 3 would contradict this. A high

IgG2a response was observed in both groups 2 (1°P/P) and 3 (1°P/2°P) after the first pregnancy and primary *C. abortus* infection, supporting the presence of a Th1 response in infected mice. Following the second pregnancy, the IgG2a response increased further in groups 2 (1°P/P), 3 (1°P/2°P) and 4 (P/1°P) in pregnant and non-pregnant mice, agreeing with previous studies (**Chapter 4**). Antibody has been suggested to be important for resistance to re-infection (Moore *et al.*, 2002; Moore *et al.*, 2003) and to enhance the immune response, and this agrees with the increased IgG2a response to *C. abortus* in group 3 mice, which were immune to secondary infection in this study. Western blotting results further supported the specificity of the IgG2a response to *C. abortus* infections and the lack of an IgG1 response in these immune mice. IgM responses were similarly higher in infected mice after the first pregnancy, and continued to increase in groups 2, 3 and 4 following the second pregnancy/inoculation. Similarly, whole IgG and IgM systemic responses have been identified in post-abortion sheep (Papp *et al.*, 1994).

One must note that the longevity of the antibody response is unknown in sheep, although it is thought to persist for at least two years in immune sheep, as they are immune to abortion in the following breeding seasons (Papp *et al.*, 1998). In contrast there is no indication of the length of the antibody response following primary infection in mice. It may be that they were immune to abortion and/or secondary experimental infection in the second pregnancy due to the shorter time between abortion after the primary infection and experimental secondary infection and the presence of antibody. Perhaps if mice had been re-infected and /or remated 1 year post-abortion as occurs in sheep, they would not be immune to abortion. This was beyond the scope of the present study.

Protein and molecular expression of IFN-γ and TNF-α were also determined, providing further evidence that a Th1 cellular immune response was present and controlled both primary and secondary *C. abortus* infection in this mouse model. Both IFN-γ and TNF-α protein responses in supernatants were higher in groups 2 (1°P/P) and 3 (1°P/2°P) than in uninfected mice (group 1; P/P) after the second pregnancy. There was a clearer distinction between higher IFN-γ responses in mice

that did not abort in the subsequent pregnancy (groups 2 and 3) to the lower IFN-γ responses of group 4 (P/1°P) mice than between TNF-α responses in the different groups, but only a proportion of samples were assessed for the latter cytokine. Increased production of cytokines in groups 2 and 3 could be linked to the presence of acquired immunity, which had not yet developed in group 4 until after day 5 p.i., These increased responses in infected than uninfected mice are similar to those found in **Chapter 4** in sera samples. Other mouse models of *C. abortus* have also found elevated IFN-γ and TNF-α responses in infected mice compared to uninfected mice (Bouakane *et al.*, 2003; Buendia *et al.*, 2004; de Oca *et al.*, 2000) as well as in other models of *Chlamydia* infections (Geng *et al.*, 1998; Qiu *et al.*, 2004; Rothfuchs *et al.*, 2004).

A reduction in mRNA expression of both proinflammatory cytokines within infected tissue sections was observed. Although an increase in mRNA expression may be expected, the lack of organisms within the tissues of groups 2 and 3 would perhaps negate the need for specific cytokine production at previous sites of infection. IFN-γ and TNF-α mRNA expression were barely detectable in tissues from group 2 (1°P/P) mice that appeared to have cleared the infection, however a specific memory response was still present in these mice, demonstrated by *in vitro* chlamydial antigen stimulation that increased cell proliferation and simultaneous cytokine production. Similarly in group 3 (1°P/2°P), where no chlamydial organisms were detected, mRNA expression of the cytokines was infrequent and reduced in comparison to previous studies (**Chapter 4**) and to the inoculum control group 4 (P/1°P) tissues, from which organisms were recovered and detected. This suggests that IFN-γ and TNF-α act locally at sites of infection in primary-infected mice (group 4) as well as specifically to *C. abortus* antigens (groups 2, 3 and 4). A memory response had developed in these immune groups, where no *C. abortus* was detected, as shown by *in vitro* stimulation with specific antigens, although this was less pronounced in primary-infected mice, where innate immunity was prevalent.

As a result of the decreased pregnancy rate, both pregnant and non-pregnant mice were examined in this study. This allowed investigation into the potential use of both

pregnant and non-pregnant mice for vaccine experiments, although a pregnant model obviously offers the advantage of using a reduction in abortion as the readout. In terms of immune responses, however, few differences were observed between them, with inflammatory cells and proinflammatory cytokines present in all infected mice. Chlamydial-specific antibody responses were also detected in both groups of mice and memory responses were detected in all infected mice, irrespective of their pregnancy status. This study therefore validates the use of both pregnant and non-pregnant mice to examine immune responses to *C. abortus* infections, although pregnant mice are ultimately better as immunity to abortion can be demonstrated, which is the outcome that is desired from successful vaccination.

Examination of immunity post-abortion revealed similarities with the ovine disease, in terms of both mice and sheep being immune to abortion in subsequent pregnancies post-abortion. This was observed in animals that were and were not administered with a secondary experimental infection. A systemic antibody response specifically to *C. abortus* was detected and a protective Th1 response was characterised. The development of other reagents for *in situ* hybridisation and detection of other immune cells by immunohistochemistry may provide further information regarding the immune response, which could then be examined in immune sheep.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

C. abortus infection of ewes results in abortion, leading to large economic losses to agricultural industries worldwide. Research has focused on the characterisation of pathology and immune responses associated with infection in the natural ovine host. The aim of this project was to develop a pregnant mouse model to assess the efficacy of vaccines, using a reduction in abortion as the readout. This will reduce costs and time and increase the scope of information that can be obtained over that arising from experiments conducted in the natural ovine host.

Initial studies involved establishment of the model by comparison of *C. abortus* infection of two mouse strains - inbred CBAs and outbred Portons - in terms of susceptibility to infection. Culture of *C. abortus* organisms from infected tissues and progression of infection were examined over the course of infection. This revealed few differences in susceptibility between the two strains. Of primary importance was the final outcome of abortion in *C. abortus*-infected pregnant mice, which is similar to that occurring in infected ewes (Buxton *et al.*, 1990; Buxton *et al.*, 2002), despite the differences in placental structure between the two species. Although sheep give birth to both dead lambs and weak live offspring that frequently die following infection with *C. abortus*, mice produced dead foetuses only. *C. abortus* progressed from the maternal to foetal side of the mouse placenta over the course of infection, which is similar to disease progression in sheep, whereby organisms migrate towards the foetus; infection established in the foetal trophoblast cells of both species. This progression was also similar to that in the pregnant mouse model developed by Buendia *et al.* (1998).

Subsequent studies of the immune response in *C. abortus*-infected mice identified characteristics of a type 1 (Th1) immune response, which is known to control chlamydial infections (Buendia *et al.*, 2002; Igietseme, 2002; Morrison & Caldwell, 2002). The inflammatory infiltrate present in infected mouse reticuloendothelial organs and placenta comprised PMNs, B cells, and $CD4^+$ and $CD8^+$ T cells, which is analogous to that seen in sheep (Buxton *et al.*, 2001). Macrophages have been described in inflammatory exudates in *C. abortus*-infected ovine placental tissue (Buxton *et al.*, 2002; Navarro *et al.*, 2004) and it is likely that these cells were

prevalent also in inflammatory infiltrates in mice. In addition, initial inflammatory responses following genital tract infection of mice with *C. trachomatis* (MoPn) are composed predominantly of PMNs, and lymphocytes and macrophages infiltrate the submucosae as the infection resolves (Morrison *et al.*, 1995; Morrison & Morrison, 2000). *Chlamydia*-specific antibodies, specifically IgM and IgG2a, were produced in response to *C. abortus* infections in mice and the latter isotype is characteristic of a Th1 immune response.

An increase in the expression of IFN-γ and TNF-α cytokines was detected in sera and by *in situ* hybridisation in liver and placental tissues in response to *C. abortus* infections. The exact mechanisms of tissue damage and abortion in *C. abortus*-infected sheep are unclear, but are probably linked to the induction of these proinflammatory cytokines in that they are not seen in normal placenta. IFN-γ expression increased around the time of abortion in mice, suggesting that it was produced to resolve the infection, as the growth of organisms is restricted by IFN-γ *in vitro* (Brown & Entrican, 1996; Byrne & Faubion, 1982). However, IFN-γ may also have contributed to abortion as Th1 cytokines are down-regulated in uninfected pregnant animals (Ragusa *et al.*, 2004) because of their potential detrimental effect on pregnancy. TNF-α levels were higher around the time of abortion in mice and TNF-α expressing cells are found in large numbers in infected ovine placentas (Buxton *et al.*, 2002). This cytokine has potential, albeit contrasting, roles in both control of the infection and the induction of abortion (Entrican *et al.*, 2004). Immunological and physiological changes, as well as the presence of the organisms during pregnancy in infected mice or sheep are likely to contribute to the final outcome of abortion.

Both non-pregnant and pregnant mice are immune to experimental secondary infection with *C. abortus* demonstrated by failure to detect the organism irrespective of pregnancy status, and the delivery of live, healthy pups by pregnant animals. This is similar to what occurs following ovine infections, where ewes have been demonstrated to be immune to experimental secondary infection (Papp *et al.*, 1994). No organisms were detected after day 3 post-secondary infection in re-infected mice

in contrast to primary infections. In future studies, tissues could be sampled on the day following secondary infection to determine whether the mice were actually susceptible to re-infection by *C. abortus.* Re-infected mice displayed a milder inflammatory response, comprised mainly of small clusters of $CD4^+$ T cells, as is observed in mice re-infected with *C. trachomatis* (Morrison & Morrison, 2000). CD4 or CD8 depleted or KO mice could be infected with *C. abortus*, as has similarly been conducted in *C. trachomatis* genital tract infections (Magee *et al.*, 1995; Morrison *et al.*, 1995) and in *C. pneumoniae* mouse infections (Rothfuchs *et al.*, 2004) to progress further understanding of the immune response. These studies would provide additional information on the relative roles of the T cell populations in immunity to the organism, to allow comparison with other mouse models (de Oca *et al.*, 2000) and other *Chlamydia* species. Neither mice nor sheep abort in the pregnancy subsequent to abortion (without secondary infection), although there appear to be differences in immunity between these species. Mice appear to have cleared the infection in this study, whereas ewes have been shown to develop chronic infections that leads to the further spread of infection in subsequent breeding seasons (Papp & Shewen, 1996).

A latent infection did not appear to develop in non-pregnant mice as occurs in sheep, highlighting possible differences in the control of *C. abortus* infection in mice and sheep. Although non-pregnant mice appeared to have either cleared or controlled the infection so that abortion did not occur, an undetectable persistent infection may have developed. In contrast, a latent infection develops in non-pregnant sheep that recrudesces to cause abortion in the following pregnancy. This re-emergence of *C. abortus* during pregnancy in sheep is likely to be the result of favourable conditions arising in the placenta, such as changes in hormonal expression and immune responses (Entrican *et al.*, 2004). A similar scenario is present in the *C. trachomatis* murine genital tract model, whereby mice naturally resolve the infection within 4 weeks without treatment and develop long-lived immunity (Barron *et al.*, 1981; Morrison *et al.*, 1995), in contrast to the multiple infections that can occur in humans that can only be controlled by antibiotic treatment. Therefore, although there are

many advantages to using chlamydial mouse models, there are clear differences in the diseases when compared to the natural hosts.

Concluding remarks and future work

Despite the increasing knowledge on the immunopathogenesis of *C. abortus* infections, the underlying mechanisms for abortion remain unresolved. Available evidence suggests that a combination of hormonal changes, inflammatory responses and pathological damage compromise survival of the foetus in infected mothers. In infected sheep, progesterone levels decrease and this is considered to play a role in abortion, as this hormone is required for the maintenance of normal pregnancy (Buxton and Henderson, 1999). It would be interesting to monitor the changes in hormonal levels during *C. abortus* infection in pregnant mice in future studies to determine if there are any clear patterns associated with abortion, as occurs in sheep.

Although both sheep and mice abort following infection with *C. abortus* the mechanisms of abortion may not necessarily be the same. Owing to their different lengths of gestation, placental structures and immune responses, the extent of pathological damage and therefore the presence of immune cells, may differ between the species. Extended inflammation and necrosis in infected placentas in sheep may explain greater expression of TNF-α mRNA expression in sheep. An increased inflammatory response to this pathological damage is likely to result in more pathological changes and this may actually contribute to abortion rather than control the infection. To provide further information on the role of TNF-α, this cytokine or its receptors could be knocked out in mice (TNFR1 KOs) (Riehl *et al.*, 2004). This would reveal whether TNF-α is involved primarily in the control of chlamydial infections or in the induction of abortion, or in both, which is the more likely scenario. Similar studies could be conducted with IFN-γ gene KO or depleted mice, although many IFN-γ depletion and gene KO experiments in mice that have been infected with *C. trachomatis* have been carried out, showing its essential role in controlling infections (Cotter *et al.*, 1997; Dalton *et al.*, 1993; Wang *et al.*, 1999). McCafferty *et al.* (1994) demonstrated exacerbated pathology and increased

infection associated with *C. abortus* infections, following neutralisation of IFN-γ in mice. It would be interesting to investigate this further by knocking out IFN-γ genes (Wang *et al.*, 1999) in mice to determine the precise role of this cytokine in *in vivo* infections and to compare results with *in vitro* studies.

Development of more probes to examine the localised expression of other Th1 cytokines such as IL-12, and even Th2 cytokines, such as IL-4 and IL-10, by *in situ* hybridisation would provide further information on the balance of Th1/Th2 immune responses at sites of infection during pregnancy. Identification of such cytokines is also important in animals that are immune to abortion to provide further information on the protective immune response to *C. abortus*. Further examination of immunity to abortion and the anatomical location of *C. abortus* as well as the mechanisms responsible for control of infection during latency are also required as it may allow an earlier detection and therefore treatment of infected sheep. However, such studies would be more beneficial in the natural host as further studies are required to determine if latent infections developed in the mouse model.

Obviously there are differences in chlamydial disease in the mouse and in the natural ovine host, however primary infections in pregnant mice closely mimic those in sheep. Similarities in pathogenesis and the strong adaptive immune response generated in this pregnant mouse model following resolution of infection establishes the usefulness of the model for the further studies of protective immunity. Future studies should thus not only focus on assessment of vaccines, but on more in depth characterisation of immunity and the role of particular cell types in *C. abortus* infected mice that can then be transferred to sheep studies. This model will be of great benefit to MRI and to the chlamydial research field.

This model could be used to assess the efficacy of the two commercial vaccines available in the UK (Mydiavac and Enzovax) in addition to assessing novel vaccine candidates, to determine whether the vaccines have a similar efficacy in both sheep and mice in terms of reducing the incidence of abortion. In conclusion, the major contribution of this pregnant mouse model will allow assessment of current and

future vaccines in a quick and effective manner, reducing time and money that would be otherwise required for sheep vaccine trials.

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Immunopathology of *Chlamydophila abortus* infection in sheep and mice

K. Kerr, G. Entrican, D. McKeever, D. Longbottom *

International Research Centre, Pentlands Science Park, Moredun Research Institute, Bush Loan, Penicuik, Midlothian, Edinburgh EH26 0PZ, UK

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Abstract

Chlamydophila abortus targets the placenta, causing tissue damage, inflammation and abortion (enzootic abortion of ewes). It is one of the main infectious causes of abortion in ewes, resulting in major economic losses to agricultural industries worldwide. Although ruminants and pigs are the principal hosts, humans are also susceptible to infection. Control of disease requires a host inflammatory response, which is likely to contribute to pathology and abortion. Mouse models have been widely used to provide insight into the role of specific immune cells in controlling infection and disease. The use of such model systems for investigating the mechanisms of abortion, latency, persistence, and immunity to reinfection will result in the identification of novel vaccine control strategies for sheep.

Keywords: *Chlamydophila abortus*; Enzootic abortion of ewes; Pregnant mouse model; Proinflammatory cytokines; Pregnancy hormones

1. Introduction

Chlamydiae are obligate intracellular bacteria that infect epithelial cells and monocyte/macrophages of a wide host range, resulting in a broad spectrum of diseases (Table 1) (Longbottom and Coulter, 2003; Schachter, 1999; Storz, 1988). Following reclassification in 1999, the order *Chlamydiales* now comprises four distinct families, namely the *Simkaniaceae*, *Waddliaceae*, *Parachlamydiaceae* and *Chlamydiaceae* (Everett et al., 1999). The *Chlamydiaceae* are divided into two genera, *Chlamydia* and *Chlamydophila,* and nine species (Table 1). This classification is based on limited phenotypic, morphological and genetic criteria (Corsaro et al., 2003; Everett et al., 1999).

Chlamydiae undergo a biphasic developmental cycle that consists of two distinct morphological forms, the elementary body (EB) and the reticulate body (RB), which are specifically adapted to extracellular and intracellular environments, respectively (Longbottom and Coulter, 2003). The EB, which is the infectious form of the bacterium, is small in size (200–300 nm in diameter) and metabolically inactive. EBs give rise to primary infection of susceptible hosts by attaching to the surface of mucosal epithelial cells. Upon entry, the EB, which resides within an intracytoplasmic inclusion, transforms into the larger (500–1000 nm) RB, which is non-infectious and metabolically active. The RB multiplies by binary fission, rapidly filling the inclusion, which increases in size. Towards the end of the developmental cycle, the RBs transform back into EBs, which are then released by lysis or exocytosis and go on to infect neighbouring cells (Beatty et al., 1994).

Chlamydiae comprise an exceptionally diverse group of organisms, infecting a wide variety of hosts and causing a broad range of diseases. This paper reviews the

* Corresponding author. Tel.: +44 1 31 4456 136; fax: +44 1 31 4456 235.
E-mail address: david.longbottom@mri.sari.ac.uk (D. Longbottom).

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Table 1
The family *Chlamydiaceae*

Species	Host	Disease/symptoms
Chlamydia trachomatis	Humans	Chronic conjunctivitis (trachoma) Sexually transmitted disease; pelvic inflammatory disease; infertility
Chlamydia muridarum	Mice, hamsters	Respiratory and genital tract infection (model for *C. trachomatis* infection in humans)
Chlamydia suis	Pigs	Intestinal, respiratory and reproductive disease
Chlamydophila pneumoniae	Humans	Pneumonia, bronchitis, pharyngitis; also associated with atherosclerosis, reactive arthritis and asthma
Chlamydophila psittaci	Birds, poultry	Respiratory disease (zoonotic pathogen)
Chlamydophila abortus	Ruminants, pigs	Abortion (zoonotic pathogen)
Chlamydophila pecorum	Ruminants, pigs	Enteric disease; pneumonia; conjunctivitis; polyarthritis; metritis; and encephalomyelitis
Chlamydophila felis	Cats	Conjunctivitis (probable zoonotic pathogen)
Chlamydophila caviae	Guinea pigs	Ocular and genital tract infection (model for *C. trachomatis* infection in humans)

immunopathology of *Chlamydophila abortus* in sheep and mice.

2. Ovine enzootic abortion and transmission of infection

C. abortus (formerly *Chlamydia psittaci* serotype 1) infects the placenta, causing a disease in sheep known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA) (Aitken, 2000; Longbottom and Coulter, 2003). It is the most common infectious cause of abortion in lowland flocks intensively managed at lambing time, and has a major economic impact on agricultural industries worldwide. Although there are no recent figures on the economic impact of EAE in the UK, losses have previously been estimated at £15–20 million per annum (Wood, 1992). The organism causes a similar disease in goats, but infections of cattle, pigs and horses are thought to be less common. Infection can also be transmitted to humans, where it is of particular concern to pregnant women (Longbottom and Coulter, 2003).

The main routes of transmission of *C. abortus* are through ingestion of organisms shed in vaginal fluids and placental membranes at the time of abortion or lambing, or through inhalation of aerosols from the environment. EBs can remain viable in the environment for several days, depending on environmental conditions (Aitken et al., 1990). This is due to the rigidity of the cell envelope, which is both osmotically stable and poorly permeable. There is some evidence of venereal transmission (Papp and Shewen, 1996), but this route is unlikely to contribute much to the epidemiology of EAE in the UK, because there is little contact between ewes and rams during the lambing season (Appleyard et al., 1985). Another potential route of transmission is through direct infection of the foetus via the placenta, although again it is unclear what contribution this might make to the epidemiology of EAE (Buxton et al., 1990, 2002).

An initial outbreak within a flock that may give rise to only a few abortions can lead to over 30% of the ewes aborting or giving birth to stillborn or weak offspring in the following year (Aitken, 2000). In subsequent lambing seasons the incidence of abortion is likely to remain at 5–10% if affected animals are left untreated.

3. Clinical symptoms

Infection of pregnant ewes with *C. abortus* up to 5–6 weeks prior to parturition leads to clinical disease, and results in either abortion in the final 2–3 weeks of gestation, or the birth of stillborn or weak lambs that frequently die in the first few days of their life (Aitken et al., 1990). Infection of ewes within the last 5–6 weeks of pregnancy often leads to the development of a latent infection, where ewes appear to be uninfected until the next lambing season (Aitken et al., 1990). Surviving lambs born to infected mothers may be affected by EAE in their first pregnancy (Wilsmore et al., 1990).

Persistent, subclinical or latent states can be maintained for several months following infection of non-pregnant ewes, probably under control of the pro-inflammatory cytokine interferon-gamma (IFN-γ) (Brown and Entrican, 1996), before the onset of pregnancy triggers bacterial multiplication (McCafferty, 1990). *C. abortus* is undetectable by any means in latently infected ewes (Jones et al., 1995), and it is not until around day 90 of gestation that the organisms are reactivated in some way, resulting in clinical expression in the placenta, and ultimately abortion.

Although, there are generally no premonitory indications of the impending abortions, vaginal discharges can be observed for up to 48 h prior to lamb loss. These discharges can continue for 2–3 weeks, adding to the

environmental spread of infection (Nietfeld, 2001). Following abortion, ewes develop protective immunity that prevents *C. abortus*-induced abortions in subsequent pregnancies. However, immunity is not necessarily sterile, as some ewes are reported to continue to shed infectious organisms seasonally for up to three years, increasing the spread of disease (Papp et al., 1994). Most ewes remain reproductively sound and can continue in subsequent breeding programmes.

4. Immunopathogenesis

One of the most fascinating aspects of *C. abortus* infection is the lack of any pathological changes until after day 90 of gestation (normal gestation is approximately 143 days), irrespective of the timing of infection (Buxton et al., 1990). Following an initial infection, it is thought that the organism resides first in the tonsil, from where it is disseminated by blood and lymph to other organs (Jones and Anderson, 1988). It has been suggested that *C. abortus* may reside in lymphoid organs during the latent phase of infection (Papp et al., 1993).

4.1. Placental infection

Ruminant placentation is cotyledonary, non-deciduate and synepitheliochorial (Entrican, 2002; Benirschke, 2002a). The association between maternal and foetal tissues of the ovine placenta is less intimate in comparison with other types of placentation such as the single layer of contact between maternal and foetal vascular systems that occurs in the discoid hemochorial placenta of mice and humans (Engelhardt and King, 1996). The cotyledons on the foetal side of the ovine placenta are associated with rounded elevations of the uterine endometrium called caruncles (maternal side), and this whole unit is called a placentome (Longbottom and Coulter, 2003; Studdert, 1968). Contact between the foetal chorion and maternal epithelium is maintained by microvillous interdigitation in the placentome, through penetration of chorionic villi into maternal crypts. In contrast, murine placentas are mainly composed of folded villous tissues with very thin foetal capillaries covered by the trophoblast cell layer, termed the placental labyrinth, and these are immersed in the maternal sinusoidal blood. This region constitutes the major site of maternal/foetal exchange, enhanced by maternal blood no longer being enclosed within proper arteries, so that maternal blood directly bathes the surface of the trophoblast cells i.e. hemochorial (Benirschke, 2002b; Cross et al., 2002). Despite these obvious differences between ovine and murine placentation, pregnant mouse models have been developed to investigate the role of immune cells and to assess candidate chlamydial vaccines (Buzoni-Gatel and Rodolakis, 1983; Caro et al., 2003). One of the main advantages of using mouse models is the length of the gestation period, being only 21 days in mice compared with approximately 143 days in sheep, offering a faster and cheaper means of analysing vaccine candidates.

Maternal haematomas develop in ovine placentas at around day 60 of gestation at the maternal–foetal interface in the hilus of the placentome (Longbottom and Coulter, 2003; Studdert, 1968). This results from leakage of blood at the septal tips of the maternal caruncle, and probably corresponds to the time that infectious organisms pass from mother to foetus. Following establishment of infection in the foetal trophoblastic epithelial cells, infection spreads out into the intercotyledonary regions of the chorion, resulting in epithelial damage, oedema and inflammation. This inflammation is a result of infiltration by neutrophils, macrophages, lymphocytes and plasma cells. Not all of the placentomes become infected, and the level of inflammation and necrosis in the cotyledons and membranes is variable (Buxton et al., 2002). Ultimately, the damage caused by infection in the placentomes compromises the maternal–foetal exchange of nutrients and oxygen, which contributes to foetal death and abortion (Buxton et al., 2002). Foetal infection is also possible, characterised by focal necrosis in the liver, and small foci of necrosis may be found in the lung, spleen, brain and lymph nodes (Buxton et al., 1990).

4.2. Inflammatory response

Chlamydial infection stimulates a strong inflammatory response, which is necessary to control infection, but could also lead to the pathology associated with the infection. This raises the intriguing question of whether abortion is due to the damage caused by the organisms themselves or to damage caused by the inflammatory host response.

Two inflammatory cytokines, IFN-γ and tumour necrosis factor-alpha (TNF-α), which are produced in response to infection, are thought to contribute to the pathology and also to threaten the maintenance of pregnancy (Entrican, 2002). Mononuclear cells expressing mRNA encoding TNF-α are abundant in infected arteries and arterioles, and also in the inflammatory exudates associated with the chorionic epithelium, whereas very few cells express mRNA encoding IFN-γ (Buxton et al., 2002). TNF-α is thought to play a crucial role in immunity to *C. abortus,* through the activation of phagocytic cells that rapidly degrade infected cells or extracellular EBs (Igietseme et al., 2002). A high concentration of TNF-α at the materno-foetal interface is incompatible with pregnancy (Dealtry et al., 2000) and TNF-α has also been linked to spontaneous embryo loss in pregnant mice (Yui et al., 1994). Therefore, TNF-α released in response to chlamydial infection (possibly

to the lipopolysaccharide component) may be contributing to the placental damage that leads to abortion (Buxton et al., 2002).

IFN-γ is crucially involved in controlling chlamydial infections (Brown and Entrican, 1996). It can activate the microbicidal activity of host macrophages and cause destruction of infected cells in vitro, indicating its importance in host resistance (Igietseme, 2002; McCafferty et al., 1994). It has been demonstrated that *C. abortus* multiplication can be restricted by IFN-γ in vitro in ovine cells (Graham et al., 1995). IFN-γ is expressed in placental tissue during normal pregnancy, but it can also be cytotoxic for human trophoblast cells at high concentrations (Yui et al., 1994). Therefore, an increase in IFN-γ expression in response to chlamydial infection within the placenta may be detrimental to the host tissue as well as to the invading organisms. It seems highly likely that the presence of IFN-γ in the placenta controls the growth of *C. abortus* but simultaneously impairs foetal survival by damaging the functional activities of placental trophoblast cells, which in turn contributes to abortion.

IFN-γ-gene knockout mouse models have been developed, and alongside in vitro studies, have proved to be extremely useful in demonstrating the essential role of IFN-γ in controlling all chlamydial infections (Dalton et al., 1993; Rottenberg et al., 2000; Lampe et al., 1998). Severe disease sequelae develop in mice that fail to develop an adequate T helper 1 (Th1) immune response, of which IFN-γ is the key effector (Yang et al., 1996). Cotter et al. (1997) demonstrated the importance of IFN-γ in resolving *C. trachomatis* infection in mice in which the gene for IFN-γ had been disrupted (IFN-$\gamma^{-/-}$), and in the mouse pneumonitis (MoPn) model system. Wild type mice infected with *C. trachomatis* MoPn resulted in the systemic dissemination of a small number of organisms to major organs from day 7–14 post-infection and the infection resolved within 3 weeks. In IFN-γ knockout mice, a prolonged and more pronounced systemic dissemination of *Chlamydia* was observed, as demonstrated by significantly higher numbers of organisms cultured from spleen tissue.

IFN-γ (and other cytokines) appears to be pivotal in the establishment of a persistent or latent infection in the host, suggesting that the latent phase is mediated by host cytokine production in non-pregnant ewes (Brown and Entrican, 1996; Entrican et al., 2001). A CD4 Th1 immune response is thought to primarily mediate the protective response to chlamydial infection, with IFN-γ being a major cytokine involved in the response. The anti-inflammatory cytokines, IL-4 and IL-10, down-regulate IFN-γ during pregnancy (Krishnan et al., 1996). This has yet to be proven in sheep but if this is the case, down-regulation of IFN-γ may lead to chlamydiae being reactivated from their 'latent' state, allowing colonisation of the placenta and disruption of the maternal–foetal junction, resulting in abortion.

4.3. Hormonal Influences

Hormones involved in pregnancy are likely to play a crucial role in inducing abortion and progesterone, 17β-oestradiol and prostaglandin E_2 (PGE_2) are all central to pregnancy (Entrican et al., 2001; Longbottom and Coulter, 2003). Progesterone is necessary for the maintenance of normal pregnancy and is produced in chorionic epithelial cells in the latter part of pregnancy. It then interacts with oestradiol and PGE_2 to control the onset of parturition. Progesterone levels decrease in infected placentas, whereas the concentrations of oestradiol and PGE_2 increase in the amniotic and peripheral plasma, and amniotic and allantoic fluids, respectively (Leaver et al., 1987, 1989). The changes in hormonal levels during pregnancy may increase susceptibility to infection, and promote placental infection at about day 90 of gestation (Buxton et al., 1990). Indeed, a study of *C. trachomatis* infection in rats demonstrated that progesterone enhances susceptibility to disease, whereas oestradiol decreases susceptibility (Kaushic et al., 2000). Chlamydial infection also affects the levels of all of the above hormones, and it has been suggested that this hormone imbalance is an underlying cause of abortion (Buxton and Henderson, 1999). Since multiplication of chlamydial organisms takes place in the foetal chorionic epithelial cells, the main source of progesterone in the latter stages of pregnancy, damage to this area is likely to play a role in altering the hormonal balance, which is necessary for the maintenance of normal pregnancy, resulting in premature labour.

Hormones also affect T-cell reactivity and cytokine production. For example, progesterone is involved in the development of Th2 cells that produce IL-4 and IL-5, 17β-oestradiol enhances IL-10 and IFN-γ secretion by antigen-stimulated clones and PGE_2 biases the cytokine production of dendritic cells towards IL-10, reducing IL-12 production (reviewed by Entrican, 2002). Thus, in pregnant animals, the inflammatory response due to the invading organisms in association with changes in hormone levels alters the placental environment and endangers the developing foetus (Buxton et al., 2002; Entrican et al., 2001).

5. Pregnant mouse models

Pregnant mouse models have been widely used to investigate the immunopathogenesis of *C. abortus*-induced abortion. The first *C. abortus* murine model was developed by Buzoni-Gatel and Rodolakis (1983), who demonstrated that infection of pregnant mice with *C. abortus* resulted in abortion. This showed similarities to abortion cases in small ruminants and indicated the value of mouse models for comparison of strain

virulence (Anderson, 1986; Rodolakis et al., 1989) and evaluation of vaccine candidates (Caro et al., 2001, 2003; de Sa et al., 1995; Hechard et al., 2003a,b).

Polymorphonuclear neutrophil (PMN) infiltration and necrosis of the maternal–foetal junctions are characteristic features of chlamydial infection of the ovine placenta (Buxton et al., 1990). Pregnant mouse models have been used to expand our knowledge regarding the role of these cells in the control of infection (Buendia et al., 1999). Larger numbers of chlamydiae were isolated from spleens of PMN-depleted mice than those of non-depleted mice suggesting they play an important role in controlling chlamydial infection in mice as well as in sheep. Additionally, non-depleted uteroplacental units showed massive neutrophil infiltration at the sites of infection (Buendia et al., 1998). It appears that PMNs play a role in controlling *C. abortus* multiplication in infected organs in the early stages of infection and may be an important component of the innate immune response. It is worth noting that such a massive inflammatory response is likely to impair normal placental function, contributing to abortion, as occurs during infection of the ovine placenta.

Although PMNs may not necessarily control secondary infection of *C. abortus* (de Oca et al., 2000b) they are crucial to the recruitment of other cells types that influence the production of pro-inflammatory cytokines. Recruitment of $CD4^+$ and $CD8^+$ T cells results in a cell-mediated response, which is necessary for resolution of chlamydial infection, probably via IFN-γ, a product of both cell types (Ramsey et al., 2000). A Th1 immune response, which is vital for *C. abortus* resolution, has been characterised in the pregnant mouse models, with high levels of IFN-γ detected and no IL-4 (a Th2 cytokine) identified in sera between days 3 and 7 post-infection (de Oca et al., 2000a).

Mouse models are continually providing new insights into the disease and a recent focus has been the role of natural killer (NK) cells, an important source of IFN-γ early in the immune response (Buendia et al., 2004). Increased numbers of NK cells are observed in spleen and liver tissue during infection of pregnant mice with *C. abortus,* and NK cells are also prevalent in the uterus of mice infected with MoPn (Tseng and Rank, 1998). The importance of these cells has been examined in mice depleted of NK cells. As expected, increased bacterial loads and a higher mortality rate were observed in NK-depleted mice when compared with wild-type mice following infection with *C. abortus* (Buendia et al., 2004). Serum concentrations of IFN-γ, which is crucial for resolution of infection, were considerably reduced in NK-depleted mice, suggesting that NK cells are an essential source of this cytokine during infection. Future studies of chlamydial infections and immune responses in mice will undoubtedly provide information on why abortion occurs and determine precise mechanisms of immunity that will inform development of novel control strategies for sheep infections.

6. Closing remarks

Despite the increasing knowledge on the immunopathogenesis of *C. abortus* infections, the underlying mechanisms responsible for abortion remain unclear. Available evidence suggests that a combination of hormonal changes, inflammatory response and pathological damage endanger survival of the foetus in infected mothers. Another unanswered question is that of why sheep that have aborted are resistant to abortion in subsequent pregnancies. Resolution of these issues will await further elucidation of protective immune responses. In addition, although immune animals are protected from further abortion, levels of immunity appear to vary; some sheep apparently develop sterile immunity post-abortion, others shed infectious organisms for up to three years (Papp et al., 1994). The anatomical location of *C. abortus* during latent or persistent infections remains unidentified and the mechanisms responsible for control of infection during latency remains unclear. Similarly, reasons behind recrudescence of infection during pregnancy have yet to be determined, although it is likely to be associated with changes in hormonal levels and immunosuppression. The stage of pregnancy at which reactivation occurs is also unknown, as the organism is not detected until day 90 of gestation in the placenta.

In conclusion, although much has been learned about the pathogenesis and control of *C. abortus* infection in ruminants, many questions remain unanswered. Ultimately, the identification of the specific mechanisms of acquired immunity will assist in the identification of vaccine candidate antigens, which can be assessed in both mouse models and the natural ovine host.

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